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The Role of Conserved Small Protein B Amino Acids in *trans*-Translation

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by

Daniel Patrick Dulebohn

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Abstract of the Dissertation

The Role of Conserved Small Protein B Amino Acids in *trans*-Translation

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Bacteria possess a unique salvage mechanism for rescuing ribosomes stalled on aberrant mRNAs. This salvage mechanism, termed *trans*-translation, is mediated by tmRNA (transfer-messenger RNA) and Small Protein B (SmpB). The specific and direct binding of SmpB to tmRNA has been shown to facilitate its delivery to stalled ribosomes. Once properly positioned in the ribosomal A-site, tmRNA acts as a tRNA and an mRNA to mediate the co-translational addition of a short peptide tag to the C-termini of nascent polypeptide chains, marking them for degradation by C-terminal specific proteases. The SmpB protein is conserved throughout the bacterial kingdom and contains several conserved amino acid sequence segments. I have used mutational analysis combined with *in vivo* and *in vitro* assays to investigate the functional significance of a number of highly conserved amino acids of SmpB.

I have demonstrated that amino acid residues E31, L91, and K124 play a crucial role in the tmRNA-mediated nascent peptide tagging process. My analysis suggests that

the peptide-tagging defect exhibited by these mutants is due to their inability to facilitate the delivery of tmRNA to stalled ribosomes. I have demonstrated through biochemical means that the ribosome association defect of these mutants is due to their reduced tmRNA binding affinity. Furthermore, I have investigated the interactions of SmpB-tmRNA orthologues from the thermophilic bacterium *T. tengcongensis*. My biochemical investigations demonstrate an analogous role for the equivalent *T. tengcongensis* residues in SmpB-tmRNA interactions. Taken together, these results demonstrate the functional significance of residues E31, L91, and K124 in SmpB binding to tmRNA, suggesting that this cluster of evolutionarily conserved residues might represent a core contact surface for recognition of tmRNA.

I have also investigated conserved amino acids located within the proximal and distal regions of the SmpB C-terminal tail. I have identified SmpB amino acids in these regions that are critical for a novel function of SmpB that is unrelated to tmRNA binding and ribosome association. This novel role in the mechanism of trans-translation is post ribosome association but prior to the first transpeptidation reaction of tmRNA on the ribosome.

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Chapter 1: Introduction

1.1 Introduction

Accurate and efficient translation of a messenger RNA into a protein is critical for all living organisms. Bacteria have developed a unique translational quality-control mechanism to ensure the efficiency of translation. This mechanism, known as *trans*-translation, is responsible for releasing ribosomes that have become stalled on aberrant mRNAs or a mRNA lacking an in-frame stop codon. This unique system also targets the incomplete proteins for efficient degradation as well as facilitating the decay of the aberrant mRNAs.

Trans-translation is mediated by a unique bi-functional RNA molecule, transfer-messenger RNA (tmRNA), that functions as both a tRNA and a mRNA. tmRNA has an essential protein cofactor Small protein B (SmpB) that is required for all of the functions of tmRNA. Upon ribosome stalling, a complex of tmRNA, SmpB and Elongation factor Tu recognize and bind to stalled ribosomes. tmRNA then performs its tRNA function, adding its alanine charge to the incomplete protein. The mRNA-like domain of tmRNA is then engaged, and a reading frame switch occurs allowing tmRNA to act as a surrogate mRNA. The mRNA-like domain is decoded by the ribosome allowing the addition of a degradation tag to incomplete proteins. The ribosome is then released by translation factors and the tagged protein is degraded by cellular proteases. tmRNA also facilitates the decay of the aberrant mRNAs through the coordinated action of RNase R. The aim of

my investigations has been to uncover the role of conserved SmpB amino acids and determine their role in *trans*-translation.

1.2 Prokaryotic Protein Synthesis

The ribosome-catalyzed formation of specific polypeptides translated from a template mRNA is a fundamental life process. This process can be broken down into three distinct phases, initiation, elongation and termination (Fig1.1).

The ribosome is the main component of the translational machinery. The largest known macromolecular enzyme, it is comprised of two subunits of unequal size. The small ribosomal subunit (30S) is composed of over 1500 nucleotides and 21 proteins(1-3). This subunit has key roles in translation initiation, decoding and controlling the fidelity of codon-anticodon interactions. The large ribosomal subunit is comprised of two RNA chains with a total of about 3000 nucleotides and 34 proteins. The large subunit performs peptide bond formation and channels the nascent proteins through the ribosomal exit tunnel. The assembled ribosome (70S) contains three binding sites for tRNA molecules, the A-site (aminoacyl), the P site (peptidyl) and the E site (exit site)(2, 3).

Initiation in bacteria typically involves the interaction of the 30S subunit with the Shine-Delgarno sequence on mRNA, which is complementary to the anti-Shine Delgarno sequence at the 3' end of 16S rRNA(4). The process also involves three initiation factors, IF-1, IF-2, and IF-3. IF-3 is known to bind to the 30S subunit and prevent its

association with the 50S subunit(5-8). It also helps in the selection of initiator tRNA (fMet-tRNA^{fMet}) by destabilizing the binding of other tRNAs in the P site of the ribosome(1, 2, 5-7, 9, 10). IF-2 is a GTPase that binds preferentially to fMet-tRNA^{fmet}, and its affinity for the ribosome is increased by IF-1(11). The current data indicates that IF-1 binds to the 30S A-site, IF-2 binds over the A-site, the P site is occupied by initiator tRNA (fMet-tRNA^{fmet}) bound to the AUG start codon of the mRNA, and IF-3 is in the E site(1, 2, 4, 8, 11, 12). The association of the 50S subunit to form the 70S complex is catalyzed by IF2 and the subsequent release of IF-1 and IF-3. The association of the 50S subunit forms a 70S ribosome complex with an aminoacylated fMet-tRNA^{fMet} bound to its AUG codon in the P-site and leaves a ribosomal A-site containing the next codon which serves to start the elongation phase of translation(1, 2).

Translation elongation is the process where an mRNA is decoded and the nascent polypeptides are elongated one amino acid at a time. One step in ensuring the fidelity of translation elongation is maintained by aminoacyl-tRNA synthetases catalyzing the addition of the correct amino acid to the corresponding tRNA. The aminoacylated tRNAs are then bound by elongation factor-Tu (EF-Tu), a GTPase, promoting formation of a ternary complex(2, 12-14). The ternary complex of EF-Tu-GTP-aminoacyl-tRNA is then brought to the ribosomal A-site to interact with the codon residing in the A-site. When the correct tRNA enters the A-site and the anticodon of the cognate tRNA interacts with the mRNA codon, elements within the decoding center of the ribosome (primarily rRNA nucleotides A1492, A1493 and G530) detect proper Watson-Crick base pairing between the codon and anticodon, and a conformational change in these nucleotides occurs. This conformational change is an essential signal indicating that proper

codon:anticodon interactions are present and that the correct tRNA is bound in the ribosomal A-site. This signal is subsequently communicated to the GTPase center in the large ribosomal subunit(1, 15-17). The mechanism of how this signal is transmitted from the decoding center in the 30S ribosomal subunit to the GTPase center on the 50S subunit ~70Å away is poorly understood, but some have suggested that the tRNA plays an active role in this step(14, 18). The transmission of this signal to the GTPase center of the large ribosomal subunit activates the GTPase domain on EF-Tu, resulting in the hydrolysis of GTP by EF-Tu. The EF-Tu-GDP then dissociates from the ribosome due to its reduced affinity for tRNAs. The aminoacyl tRNA then fully enters the ribosomal A-site in a process known as accommodation. The tRNA swings into the A-site, positioning the aminoacyl stem of the tRNA in the peptidyl-transferase center of the ribosome. Peptide bond formation, transferring the peptide chain to the A-site tRNA, then proceeds rapidly, leaving a deacylated tRNA in the P-site and a peptidyl tRNA in the A-site(1, 2, 12, 16, 17). Translocation of the tRNAs and mRNA is facilitated by the action of another GTPase, Elongation Factor-G (EF-G) (1-3, 19). Following translocation, the ribosome is left with a deacylated tRNA in the ribosomal E-site, a peptidyl tRNA in the ribosomal P-Site and a ribosomal A-site containing the next codon awaiting the cognate tRNA-EF-Tu-GTP complex.

Translation elongation continues until the ribosome encounters one of the three stop codons found in bacteria (UAA,UAG and UGA). Stop codons are recognized in the ribosomal A-site by one of two class 1 release factors (RF-1 recognizes UAA and UAG and RF-2 recognizes UAA and UGA)(20). The class 1 release factors mediate hydrolysis and release of the peptide from the tRNA in the P-site (21). Another GTPase, RF-3 then

binds the ribosome and induces release of RF-1 or RF-2 (15, 22). This 70S complex is disassembled by another factor, ribosome recycling factor (RRF) along with EF-G(23-25). Finally, IF-3 is required for removal of the deacylated tRNA bound to the 30S ribosomal subunit(26).

The ribosome however does not always find itself translating a mRNA with all of the requisite signals to ensure efficient translation. Gene mutation, premature transcription termination, DNA damage, mRNA damage and translational errors can all lead to the ribosome reading a mRNA lacking an in-frame stop codon. In these cases, the ribosome will fail to enter the termination phase of protein synthesis. Instead, ribosomes will continue the elongation phase until the ribosome reads to the 3' end of a mRNA and stalls. These stalled ribosomes have a number of consequences for the cell summarized in Figure 1.2A. Firstly, an mRNA is decoded by several ribosomes decoding in the 5' to 3' direction, and stalling at the 3' end of a message will result in the sequestration of multiple ribosomes that will then be unavailable for subsequent rounds of translation. Secondly, the incomplete polypeptide, if released, might be deleterious to the cell. Thirdly, the aberrant mRNA, if not removed, could result in further wasteful rounds of unproductive translation. Bacteria have evolved a unique mechanism known as *trans*-translation for dealing with the problems associated with ribosome stalling.

1.3 *Trans*-Translation

Trans-translation is mediated by a unique bi-functional RNA molecule, transfer-messenger RNA (tmRNA), that functions as both a tRNA and an mRNA. tmRNA has an

essential protein cofactor SmpB that is required for all of the functions of tmRNA. Upon ribosome stalling, the complex of tmRNA, SmpB and EF-Tu recognize and bind to stalled ribosomes. tmRNA then performs its tRNA function, adding its alanine charge to the incomplete protein. Translation switches to tmRNA, which then acts as a surrogate mRNA allowing the addition of a degradation tag to complete the proteins. The ribosome is then released by translation factors and the tagged protein is recognized and degraded by cellular proteases. tmRNA also facilitates the decay of the aberrant mRNAs through the coordinated action of RNase R. A number of factors participate in the *trans*-translation mechanism and I will describe these factors in detail before returning to a complete overview of the *trans*-translation system. See Figure 1.2B for schematic representation of the *trans*-translation mechanism.

1.3.1 tmRNA

Transfer-messenger RNA (tmRNA, also known as 10Sa RNA and SsrA RNA) is a large bi-functional RNA molecule of 363 nucleotides in *Escherichia coli*. Coded for by the *ssrA* gene, tmRNA is crucial to the *trans*-translation ribosome rescue mechanism (27-31). tmRNA has the unique ability to act as both a tRNA and a mRNA. The predicted secondary structure of tmRNA is shown in Figure 1.3 (32, 33). The 5' and 3' ends of tmRNA form a tRNA-like domain, including an acceptor stem, a T-arm, and a D-loop with no stem. Lacking an anti-codon stem loop, tmRNA has an extended disrupted stem that links the tRNA-like domain to the rest of the molecule(34-36). The extended stem is followed by a pseudoknot (PK1) that is connected to the mRNA-like domain of tmRNA.

The mRNA-like domain ends within a hairpin loop containing two consecutive UAA stop codons. The mRNA-like domain is followed by three pseudoknots that link the mRNA-like domain to the 3' end of the molecule.

The tRNA-like properties of the folded 5'and 3' end of the tmRNA molecule have been demonstrated *in vitro*(36-38). The tmRNA molecule is effectively recognized and charged by alanyl-tRNA synthetase. Proper charging of the tmRNA molecule has also been shown to be necessary for association of tmRNA with 70S ribosomes. It was also determined that the charging determinants of tmRNA could be altered, allowing for charging by histidyl-tRNA synthetase *in vitro*(34, 39). Recently, the co-crystal structure of the tRNA-like domain of tmRNA in complex with SmpB has been solved(40, 41). This structure highlights the unique nature of the tRNA-like domain of tmRNA. A traditional tRNA molecule fold conforms to the traditional "L" shaped tRNA structure. Surprisingly, the tRNA-like domain of tmRNA, instead of a traditional 90° turn between the amino acid-acceptor stem and the anticodon, has a turn of 120°(40). Interestingly, the bound SmpB is found at a 90° angle from the amino acid acceptor stem and is suggested to play a role as a mimic of a region of the variable loop of the tRNA-like domain. Modeling this unique structure of the tRNA-like domain of tmRNA and SmpB onto ribosomes also positions the C-terminal tail of SmpB in the direction of the 30S ribosomal A-site (41).

tmRNA contains a number of pseudoknots in its predicted secondary structure. The presence of these pseudoknots has been confirmed through structural probing experiments, and the structure of PK1 has been solved by NMR(35, 38, 42, 43). Although there is structural information on these elements, the role of these pseudoknots

in the mechanism of *trans*-translation remains poorly understood. PK 1 was originally shown to be required for effective aminoacylation of tmRNA as well as protein tagging by tmRNA(44). A recent report however has demonstrated that PK1 is not strictly required for the tmRNA tagging function *in vivo*. PK1 was effectively replaced by a stable stem-loop structure *in vivo*, which suggests a structural role for this region of tmRNA(45). Pseudoknots 2-4 have been less well studied and it has been shown that *in vitro* these elements can be substituted with single stranded RNA without affecting the tagging function of tmRNA(44). However, another report suggests that these pseudoknots play an important role in the maturation and folding of tmRNA *in vivo*(46). Clearly, additional experiments will be needed to better define the roles of these tmRNA elements.

The mRNA-like domain of tmRNA codes for the peptide ANDENYALLA, a ten amino acid degradation tag that is appended to incomplete peptides. This degradation tag is effectively recognized by a number of different cellular proteases including ClpXP, ClpAP, Tsp, Hflb and Lon (47-50). The presence of a protein coding region of tmRNA was originally identified when expression of IL-6 in *E. coli* resulted in IL-6 protein as well as protein fragments of IL-6 of varying lengths with an appended 11 amino acid degradation tag (51). The tmRNA model was then proposed by Keiler et al. integrating the observation that the appended sequence contained the alanine charged to tmRNA as well as the 10 amino acids derived from the mRNA-like domain of tmRNA. The original tmRNA model proposed that charged tmRNA binds to stalled ribosomes, donates its alanine charge, and then acts as a surrogate mRNA appending the degradation tag to the incomplete peptide. This allows normal release of the stalled ribosomes when the

ribosome encounters the tmRNA stop codons, and the released proteins tagged at their C-termini are recognized and quickly degraded by cellular proteases(52).

1.3.2 SmpB

Small Protein B (SmpB) is a small (160 amino acids in *E. coli*), basic (pI~10) protein that is a required factor for *trans*-translation. SmpB binds to tmRNA with high affinity and specificity for the tRNA-like domain. (53, 54). SmpB is required for the stable association of tmRNA with 70S ribosomes(40, 55, 56). SmpB, although not strictly required for proper amino-acylation of tmRNA, greatly enhances the ability of tmRNA to be aminoacylated *in vitro*(41, 53). This is supported by a recent co-crystal structure that suggests that a region of SmpB protein is positioned in proximity to the tRNA acceptor stem, stabilizing the acceptor stem and presumably increasing aminoacylation efficiency(41). The relative cellular concentration of SmpB and tmRNA has been determined *in vivo* to be 1:1. It was also determined that the ratio of SmpB:tmRNA on the ribosome is 1:1 (57, 58). It has been reported that while transiting the ribosome the tmRNA:SmpB concentration on the ribosome remains 1:1, suggesting that SmpB does not dissociate from tmRNA during *trans*-translation(58).

Recently, a great deal of structural information on SmpB and tmRNA has become available. NMR structures from *Aquifex aeolicus* and *Thermus thermophilus* as well as two co-crystal structures of SmpB bound to the tRNA-like domain of tmRNA have been solved(40, 41, 55, 59). In all of these structures, the core structure of SmpB protein is similar. SmpB is an anti-parallel β-barrel consisting of an oligonucleotide-binding fold

that is capped with two or three helices. Analysis of these structures reveals that SmpB has clusters of highly conserved residues on two distinct faces of the protein(55, 60). One of these surfaces consists of residues that are responsible for binding to the tRNA-like domain at the 3' end of the D-loop. Mutations to these residues impair the protein's ability to bind tmRNA and deliver it to stalled ribosomes(54). Conserved amino acids located on the surface of SmpB, opposite the tmRNA binding face, have been suggested to play a role in binding stalled ribosomes(61). Although hydroxyl-radical probing experiments do place these residues in the proximity of the ribosomal A-site, their role in ribosome association has not been elucidated (62).

Interestingly, none of the structural models to date have been able to determine a structure for the SmpB C-terminal tail. Based on structural predictions, it has been suggested that the C-terminal tail forms an alpha helical structure, but this has yet to be shown by biochemical means(63). The C-terminal tail of SmpB, though unstructured in solution, performs a novel function that is critical for supporting the first trans-peptidation reaction of tmRNA on the ribosome(64). SmpB variants lacking the C-terminal tail or harboring mutations to highly conserved amino acids within the C-terminal tail fully support tmRNA binding and tmRNA-SmpB ribosome association but fail to add the tmRNA tag to incomplete proteins(64).

1.3.3 Additional Components of the tmRNA *trans*-translation system

A number of cellular factors beyond tmRNA and SmpB participate in various stages of the *trans*-translation mechanism, including charging tmRNA, facilitating

tmRNA ribosome interactions, facilitating the decay of the tmRNA-tagged proteins, and promoting decay of the aberrant mRNA. Alanyl tRNA synthetase (Ala-RS) and elongation factor-Tu (EF-Tu) are two factors essential for the activity of tmRNA. Due to the structure of tmRNA, namely an amino acid acceptor stem ending in CCA at the 3' end and a G•U wobble base pair at position 3 and 357, tmRNA is effectively recognized and charged with alanine by Ala-RS(65-67). Ala-RS recognition and charging is essential for the function of tmRNA; non-aminoacylated tmRNA is not recognized by EF-Tu and does not associate with 70S ribosomes(68). EF-Tu binds to the tRNA-like domain of tmRNA in the amino acid acceptor arm and protects the ester linkage of the aminoacylated molecule from hydrolysis. EF-Tu is also important for delivery and initial binding of tmRNA to stalled ribosomes(69). The first transpeptidation reaction allowing the addition of the tmRNA alanine charge can occur in the absence of EF-Tu *in vivo* but at considerably reduced rates as compared to reactions where EF-Tu is present(70).

One of the key observations made by Keiler et al. was that the amino acid tag appended to IL-6 from tmRNA was recognized by cellular proteases as a signal for the degradation of the tagged product. Since this initial observation, several proteases have been identified that recognize and degrade tmRNA-tagged proteins. The proteases identified to date are Tsp, FtsH, ClpAP, ClpXP and Lon(47, 48, 50, 71, 72). Tsp is a periplasmic energy-independent protease that recognizes the tmRNA degradation tag on proteins exported to the periplasm. FtsH is a membrane-bound ATP-dependant protease that acts on tmRNA-tagged substrates. The ClpAP and ClpXP proteases are multicomponent enzymes consisting of the ClpP peptidase and either the ClpA or ClpX chaperones. The ClpA and ClpX components act by recognizing the tmRNA tag,

unfolding the tagged protein and translocating the protein into the ClpP protease component (72-74). Degradation of tmRNA-tagged proteins by the ClpXP protease complex is stimulated by an adaptor protein SspB. SspB binds to the tmRNA tag and delivers the tagged proteins to the ClpXP protease. SspB then tethers the protein substrate to the ClpXP protease complex for efficient degradation(49, 75-77). Recently, the ATP-dependant protease Lon was pulled out of a genetic screen designed to find factors involved in *trans*-translation(47). Lon was shown to preferentially degrade tmRNA-tagged proteins *in vivo* and contribute to the degradation of tmRNA-tagged proteins *in vivo*. The authors suggest that, although the ClpXP complex makes the largest contribution to the degradation of tmRNA proteins in *E. coli*, Lon protease might play a significant role in bacteria lacking the ClpXP machinery(47).

Targeted degradation of the aberrant mRNAs responsible for ribosome stalling is an important facet of the *trans*-translation mechanism. A critical component of this mRNA degradation pathway is a 3'-5' exoribonuclease, RNase R(78). The suggestion that RNase R might play a role in the degradation of damaged mRNAs that elicit *trans*-translation occurred when RNase R was found in a ribonucleoprotein complex containing tmRNA and SmpB(79). It was then shown that degradation of the damaged mRNA is a tmRNA and SmpB-dependent process, suggesting that *trans*-translation must be active to target these mRNAs for degradation. In a series of elegant experiments, it was demonstrated that RNase R was specifically responsible for degradation of the aberrant mRNAs that cause ribosome stalling(80). The timing of mRNA release and subsequent degradation by RNase R when *trans*-translation is engaged is currently unknown.

1.4 Physiological Relevance

The *smpB* and *ssrA* (tmRNA coding) genes are present in all bacteria examined to date(32, 81-83). The SmpB-tmRNA translation quality-control system has been shown to be important for both survival and pathogenesis of several different bacterial species. The SmpB-tmRNA quality-control system is essential in pathogenic *Neisseria gonorrheae*, *Mycobacterium genitalium*, and *Mycobacterium pneumonia(84, 85).* Although found throughout Eubacteria, the SmpB-tmRNA system is not essential for growth of *E. coli*, *Salmonella typhimurium*, *Bacillus subtilis*, *Yersinia pestis*, or *Yersinia pseudotuberculosis* under ideal conditions(53, 86-88). When lacking the SmpB-tmRNA quality-control system, bacteria are more sensitive to adverse conditions such as oxidative stress and sublethal concentrations of translation-specific antibiotics, and bacteria also show a slow recovery from carbon starvation(86, 89-92). *S. typhimurium* and *Y. pseudotuberculosis* lacking this system are unable to replicate within macrophages, presumably due to an inability to deal with the harsh environment presented there. The role of the SmpB-tmRNA system in the virulence mechanisms of *Y. pseudotuberculosis* has been particularly well described. Most interesting was the finding that *Y. pseudotuberculosis* lacking the SmpB-tmRNA translational quality-control mechanism was unable to cause lethal disease in a mouse infection model. *Y. pseudotuberculosis* lacking the SmpB-tmRNA quality-control system was severely defective in the ability to express and secrete Yersinia outer proteins (Yops), effector proteins involved in virulence(86).

The SmpB-tmRNA system also plays a role in modulating gene expression through maintenance of intracellular concentrations of regulatory factors, including transcriptional activators and repressors(93, 94). Consistent with this role, a number of regulatory proteins have been shown to be tagged by the SmpB-tmRNA system, including LacI repressor, the λ-cI repressor, YbeL, GalE, and RbsK(95-97). In the case of the LacI repressor, a negative feedback loop is thought to maintain the optimal concentration of LacI needed in the cell. Consistent with this hypothesis, cells lacking tmRNA exhibit a delay in the induction of the *lac* operon(95). Another example of a regulatory role for SmpB-tmRNA-mediated tagging comes from studies in *Caulobacter cresentus*. Optimal timing between the degradation of a specific response regulator, CtrA, and initiation of DNA replication is influenced by tagging and degradation of the response regulator(98).

1.5 Translation Surveillance by tmRNA

The conformational state of the ribosome or the signal that elicits specific recognition by the tmRNA-SmpB-EF-Tu complex is still unknown. It is clear that recognition of stalled ribosomes does require tmRNA-SmpB-EF-Tu-GTP in a stable complex(53, 57, 69). Beyond mRNAs lacking an in-frame stop codon, a variety of other sequence elements in an mRNA or in the sequence of the newly synthesized protein can lead to stalling of the ribosome at internal sites. A ribosome translating an mRNA containing a sequence of multiple rare codons, or where properly charged tRNAs are unavailable, leads to tagging by the tmRNA system(99, 100). Also, mRNAs ending in

weak stop codons or where release factors have been depleted can also lead to tmRNA tagging(100-104). Some protein sequences, such as the SecM-arrest sequence can also result in the recruitment of the tmRNA system. This particular amino acid sequence interacts with the ribosome exit tunnel and slows the rate of translation by the ribosome, resulting in tagging by the tmRNA system(105-107). When these internal stalling events occur, the mRNA being decoded must still reside within the ribosomal A-site(108, 109). How does the tmRNA-SmpB-EF-Tu complex recognize these substrates? How does the tmRNA-SmpB-EF-Tu complex fit within the ribosomal A-site along with the aberrant mRNA? It has become increasingly clear that these ribosome-stalling events at internal sites lead to mRNA cleavage in the ribosomal A-site that is translation-dependent but SmpB and tmRNA-independent(110-113). The endonuclease involved in this event has not been identified. It has been suggested that the ribosome or a toxin-antitoxin pair might catalyze this reaction(109, 113-116). After mRNA cleavage and release of the 3' end of the message from the ribosome, the ribosome is left with a peptidyl-tRNA in the ribosomal P-site and an empty A-site that would be a substrate for the tmRNA-SmpB-EF-Tu complex. How cleavage and release of the 3' end of the mRNA occurs remains an open question.

1.6 Complete Model of the tmRNA Mediated *Trans*-Translation System

The complete *trans*-translation system for dealing with aberrant mRNAs is depicted in Figure 1.4. The process of releasing stalled ribosomes begins with the high affinity binding of SmpB to the tRNA-like domain of tmRNA. This is followed by the efficient

aminoacylation of tmRNA by alanyl tRNA synthetase. Aminoacylated tmRNA is then recognized by EF-Tu-GTP and allows formation of the ribosome-recognition complex containing tmRNA-SmpB-EF-Tu-GTP. This complex then binds to stalled ribosomes and, through contacts made by either tmRNA, SmpB or both, the GTPase domain on EF-Tu is activated and allows full accommodation of the tmRNA-SmpB complex into the ribosomal A-site. This allows tmRNA to donate its alanine charge to the incomplete peptide, and the ribosome switches from the damaged message to the mRNA-like portion of tmRNA. The ribosome begins decoding the mRNA-like domain of tmRNA and, likely during an early stage of tmRNA decoding, the damaged mRNA is released from the ribosome. This message is then specifically targeted and degraded by RNase R. The ribosome continues decoding the mRNA-like domain of tmRNA, appending the degradation tag to the incomplete protein. The ribosome continues until encountering the in-frame stop codons of tmRNA, promoting release of the tagged protein as well as the ribosome by the action of ribosome release factors. The released protein is targeted and quickly degraded by various C-terminal specific proteases. This translation quality-control system effectively deals with all of the problems associated with ribosomes that stall on aberrant messages by promoting release of stalled ribosomes and eliciting the specific degradation of both the incomplete protein and the aberrant mRNA. My investigations have focused on the protein factor SmpB, and I have elucidated the role of several conserved SmpB amino acids in *trans*-translation.

1.7 Figures

Figure 1.1

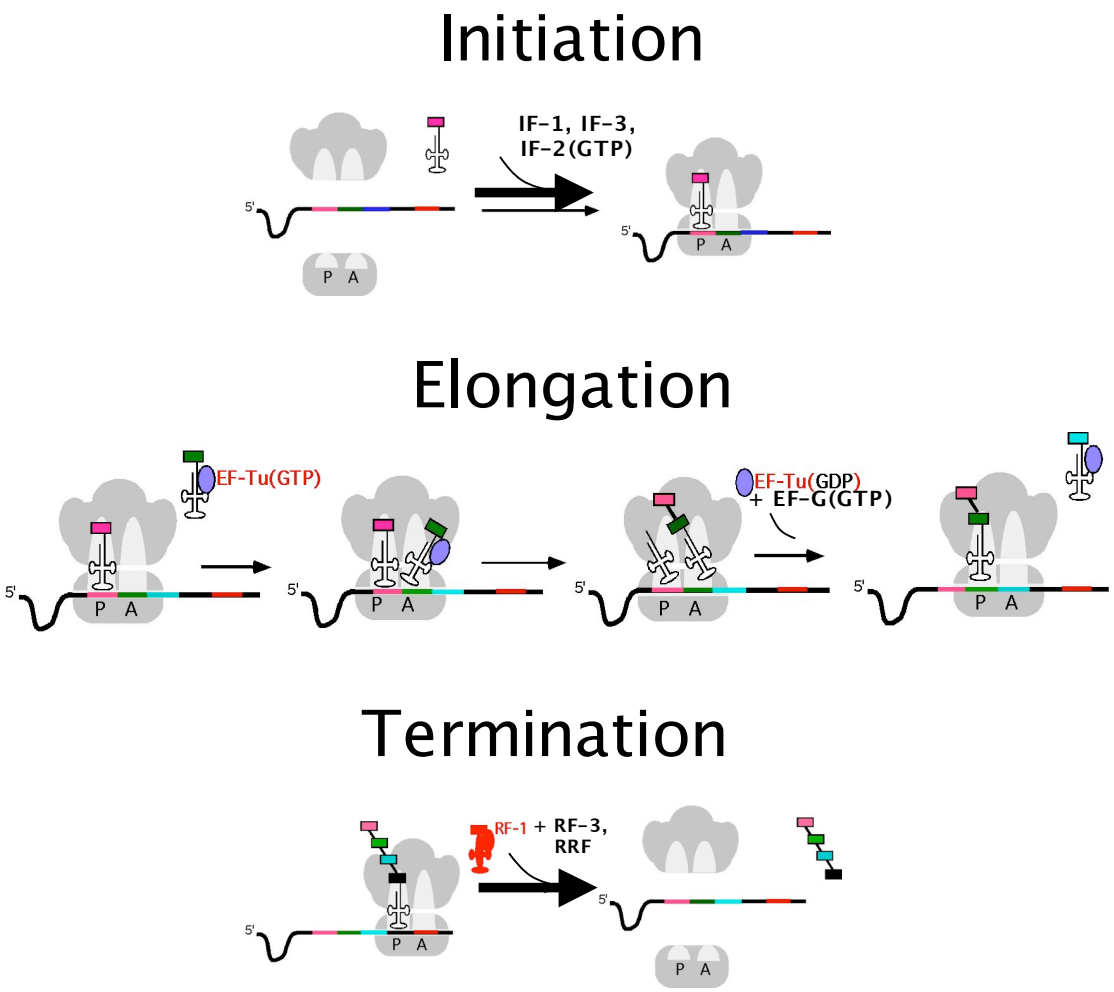


Figure 1.1: *Outline of the mechanism of protein synthesis.* Protein synthesis may be divided into translation initiation, elongation and termination phases. See text for details. Figure kindly provided by Thomas R. Sundermeier

Figure 1.2

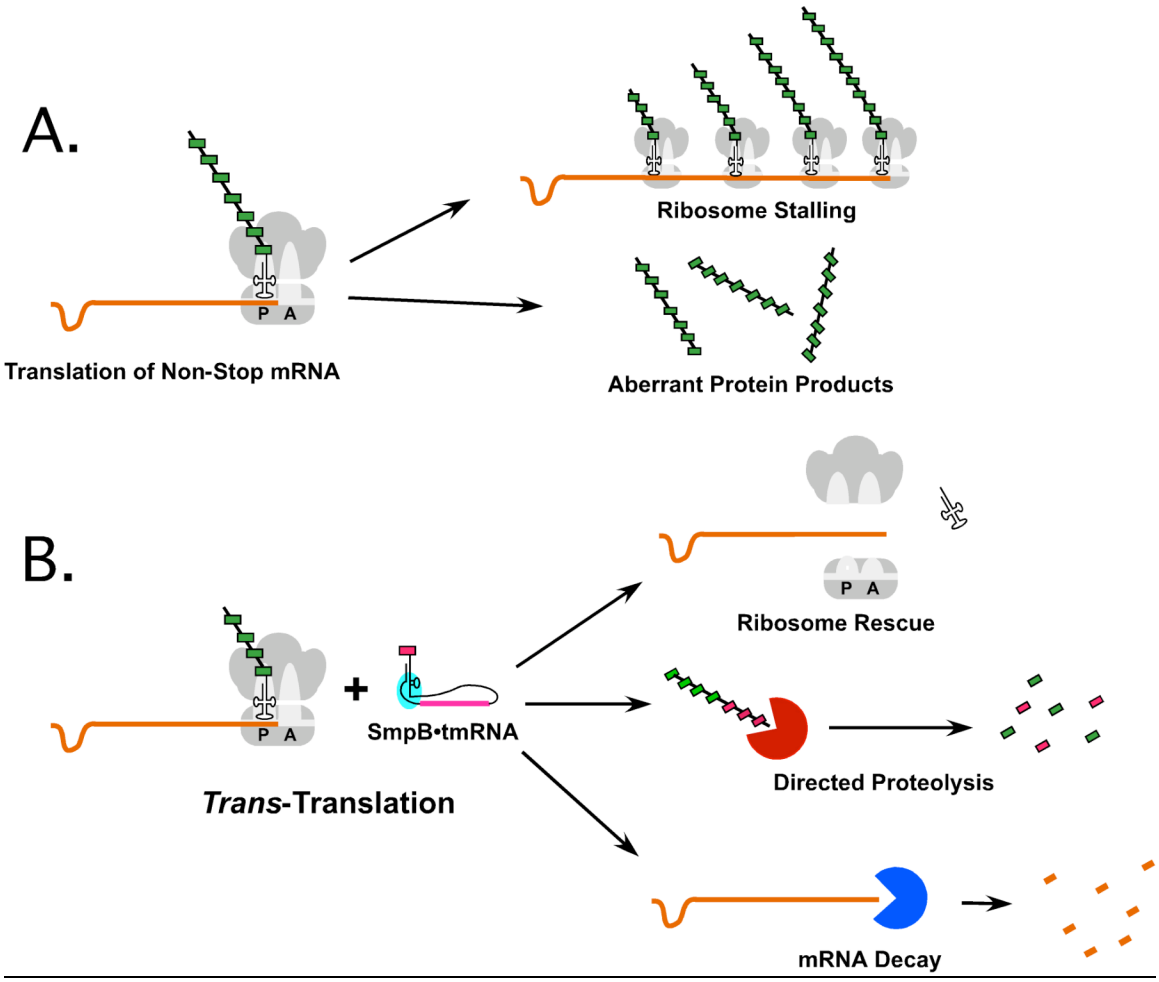


Figure 1.2: A.) Problems associated with stalled ribosomes, sequestration of ribosome and release of aberrant protein products. **B.)** The tmRNA-SmpB mediated translation quality-control system addresses the three major problems associated with stalled ribosomes through release of stalled ribosomes, directed proteolysis of the aberrant polypeptide and facilitating the decay of the damaged message. Figure kindly provided by Thomas R. Sundermeier

Figure 1.3

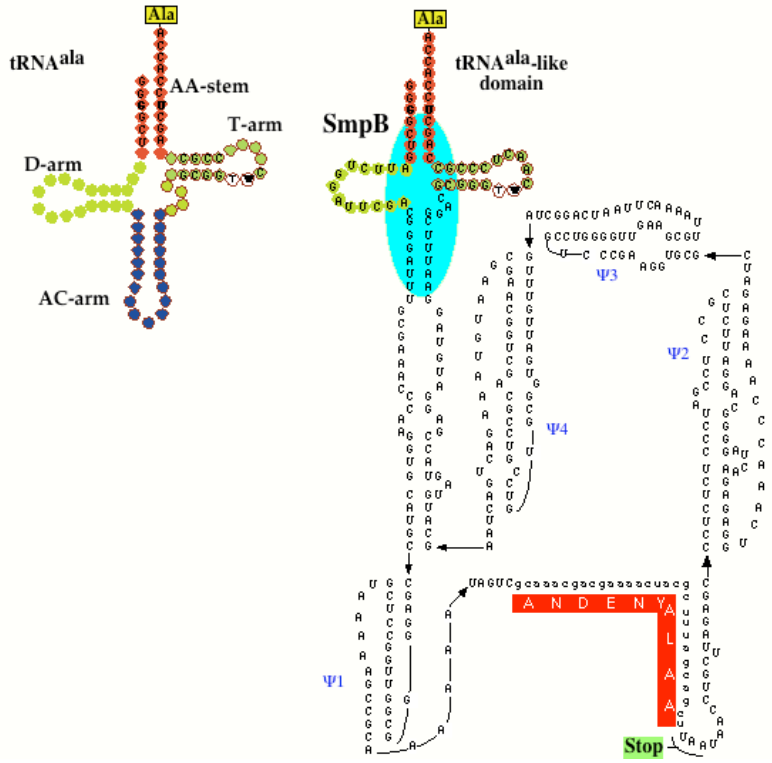


Figure 1.3: Predicted Secondary Structure of tmRNA. $tRNA^{Ala}$ is shown for comparison to show the similarities between the tRNA-like domain of tmRNA and traditional tRNAs. SmpB is shown in cyan bound to the tRNA-like domain of tmRNA. Figure adopted from Williams et al. 1996.

Figure 1.4

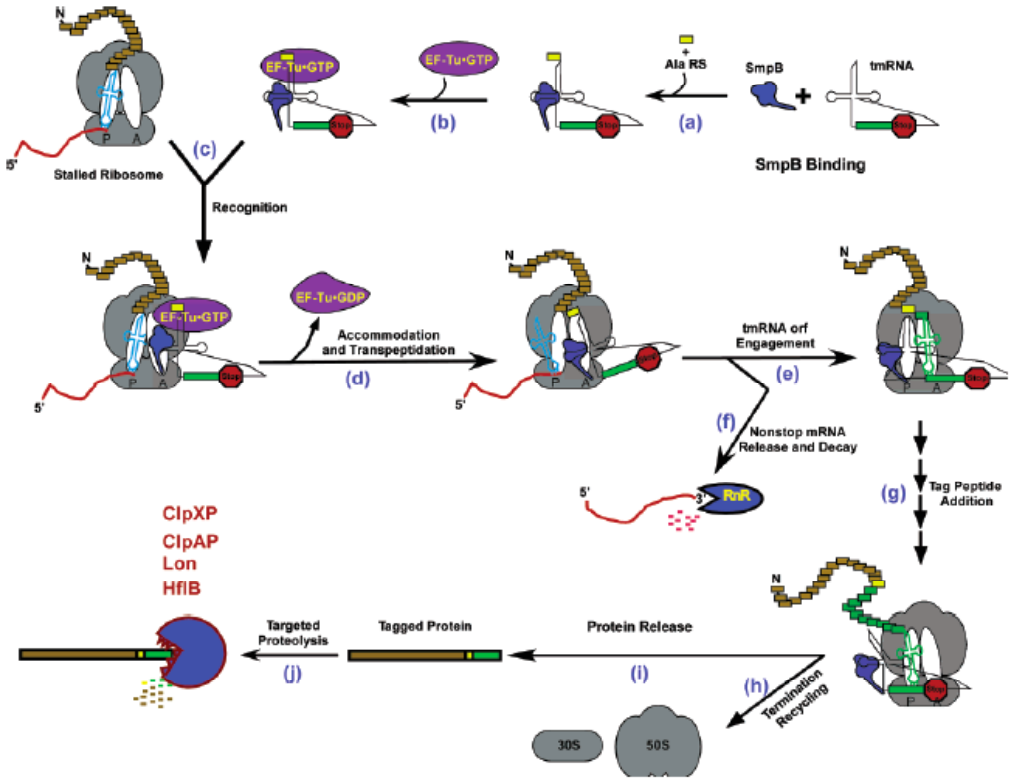


Figure 1.4: Overview of the Model of the Translation Quality Control System. a.) SmpB binding to the tRNA-like domain of tmRNA and a.) aminoacylation by Alanyl tRNA synthetase. b.) Binding of EF-Tu-GTP and formation of a ribosome recognition complex then c.) recognizes and stably associates with stalled 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 trans-peptidation. e.) The mRNA-like domain is then decoded, f.) the damaged message released and 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)

Chapter 2: Identification of Amino Acids within the SmpB C-terminal tail that are Critical for the Function of SmpB

*** The work contained within this chapter has been published with the work of Thomas R. Sundermeier in The Proceedings of the National Academy of Sciences of the United States of America in 2005(64).**

2.1 Summary

SmpB is a requisite factor in the mechanism of *trans*-translation and is required for all of the known functions of tmRNA. The defined functions of SmpB are binding tmRNA and promoting stable association of the tmRNA-SmpB complex with 70S ribosomes. Using site-directed mutagenesis of SmpB protein, I have identified SmpB amino acids in the distal region of the unstructured SmpB C-terminal tail that are critical to support the tagging function of *trans*-translation. I show by biochemical means that mutation of these critical SmpB amino acids does not affect the previously known functions of SmpB, namely, tmRNA binding and ribosome association. These amino acids must therefore be involved in a novel function of SmpB protein that is post-ribosome association and prior to transpeptidation of the tmRNA alanine charge to incomplete proteins. These critical amino acids are likely involved in interactions on the ribosome that are necessary for accommodation and transpeptidation of the tmRNA-SmpB complex in the ribosome.

2.2 Introduction

Small protein B (SmpB) is required for all known activities of tmRNA although its role in *trans*-translation beyond stably binding tmRNA and facilitating tmRNA delivery to stalled ribosomes has not been defined. There has been a great deal of structural information regarding SmpB that has become available, including two NMR structures as well as two co-crystal structures containing SmpB bound to the tRNA-like domain of tmRNA(40, 41, 55, 59). The core structure of SmpB is similar in all of these structures, however, the C-terminal tail of SmpB remains unstructured in solution in all of these structures.

In this study, I have analyzed the functional relevance of a number of highly conserved residues within the C-terminal tail of SmpB. I have identified SmpB amino acids that are critical in supporting the tmRNA tagging function. Mutation of these critical residues, however, does not affect SmpB-tmRNA binding *in vitro*, nor do they affect the ability of the tmRNA-SmpB complex to recognize stalled ribosomes. Interestingly, mutations of these highly conserved C-terminal tail amino acids yield a phenotype similar to SmpB C-terminal tail deletion mutants. I propose that these highly conserved amino acids within the SmpB C-terminal tail play an important role in *trans*-translation that is post-ribosome association but prior to the addition of the tmRNA-alanine charge to incomplete proteins.

2.3 Experimental Procedures

The generation of strains W3110ΔsmpB1, W3110 Δ*smpB*(DE3), and W3110Δ*ssrA*::CAT, and the plasmid pKW24, pPW500 and pETBA have been described previously (53). The plasmid pTC9, for overexpression of terminal tRNA nucleotidyl

transferase, was a kind gift from Dr. Charles McHenry (UCHSC). The $SsrA^{H6}$ gene was amplified by PCR from pKW24. This PCR product was used as mutagenic primer to convert pETBA to $pETBA^{H6}$. SmpB mutations were generated using the QuickChange method (Stratagene) to alter the SmpB coding sequences of pETBA and $pETBA^{H6}$. All mutants were confirmed by sequencing of the smpB gene in each one of the corresponding plasmids

Protein Purification

$SmpB^{WT}$, $SmpB^{K138A/R139A}$, $SmpB^{D137A/K138A/R139A}$, and $SmpB^{R139E}$ were overexpressed from Δ*smpB*(DE3)/pLysS/$pETBA^{H6}$ cells using 1mM IPTG induction. Cells were collected and resuspended in lysis buffer I [50mM Tris (pH7.5), 1M NH_4Cl, 10mM $MgCl_2$, 10mM Immidazole, and 2mM β-mercaptoethanol]. Cells were lysed by sonication and cell debris was removed by centrifugation. SmpB was purified from the supernatant using affinity chromatography over Ni-NTA beads (Qiagen). The Ni-NTA eluted protein was further purified by ion exchange chromatography on a MonoS (HR 10/10) FPLC column (Amersham) using a linear salt gradient. Buffer IA contained 50mM Tris (pH8), 50mM NH_4Cl, 5mM $MgCl_2$, and 2mM β-mercaptoethanol, and Buffer IB contained 50mM Tris (pH8), 1.0 M NH_4Cl, 5mM $MgCl_2$, and 2mM β-mercaptoethanol. Purified proteins were analyzed by electrophoresis, flash frozen in liquid nitrogen and stored at -80°C.

Terminal tRNA nucleotidyl transferase was overexpressed from DH5α/pTC9 cells using 1mM IPTG induction for 3hrs. Cells were harvested and resuspended in 30mL of lysis buffer II containing 50mM Tris (pH 7.5), 150mM KCl, 10 mM $MgCl_2$, and

2mM βMercaptoethanol. Cells were lysed by sonication and cell debris was removed by centrifugation. Solid ammonium sulfate was added to the lysate to a final concentration of 30% (w/v) and the solution was stirred at 4°C for 1hr. The sample was then centrifuged at 30,000xG for 30 min. The ammonium sulfate concentration in the Supernatant was then raised to 45% and processed as before. The 45% pellet was resuspended in Buffer IIA [20mM potassium phosphate (pH7.4), 1mM $MgCl_2$, 1mM DTT, 0.1mM PMSF, and 5% glycerol (v/v)]. The resuspended protein sample was then loaded onto an FPLC monoQ (HR 10/10) column (GE Healthcare), washed with buffer IIA and eluted with a linear pH gradient of 100% Buffer IIA to 10% Buffer IIB [200mM potassium phosphate (pH 4.5), 1mM $MgCl_2$, 1mM DTT, 0.1mM PMSF, and 5% glycerol (v/v)].

In vitro Transcription and RNA Labeling

tmRNA^{113} was transcribed *in vitro* from PCR products. Two primers representing the 5' and 3' ends of the tmRNA^{113} coding sequence (with overlapping complementary sequence) were extended using Pfu Turbo DNA polymerase (Stratagene). This PCR extension product was used as template in a second PCR reaction with 5' primer designed to add a T7 promoter sequence immediately 5' of the first transcribed base. The resulting dsDNA product was gel purified and used as template for *in vitro* transcription using T7 RNA Polymerase (USB). Transcription reactions were treated with DnaseI (Amersham-GE Healthcare) to digest template DNA, and RNA was purified on a 12% denaturing polyacrylamide gel (SequaGel, National Diagnostics). tmRNA^{113} was labeled at its 3'end using purified terminal tRNA nucleotidyl transferase. The labeling reaction (100uL)

contained 50mM Glycine (pH9), 10mM $MgCl_2$, 1mM $NaPP_i$, approximately 100pmoles of $tmRNA^{113}$, 10mM $\alpha^{32}P$ ATP (3000 Ci/mmol), and 1μg of purified terminal tRNA nucleotidyl transferase. Reactions were incubated at 37°C for 15min, then 5 units of yeast pyrophosphatase was added and reactions were incubated at 37°C for an additional 1min, before reactions were quenched using TriReagent LS (MRC).

The probe used for Northern Blots was biotinylated full length tmRNA dsDNA. The *ssrA* gene was amplified from pETBA by PCR and SsrA dsDNA was gel purified and biotinylated using a psoralen biotin labeling kit (Ambion).

Endogenous tmRNA-His-6 Tagging Assays

The endogenous tagging assay protocol was modified from Karzai et al. (6). 50mL cultures of either Δ*ssrA*::CAT, or Δ*smpB* (DE3) with $pETBA^{H6}$ were grown to late log phase (when Δ*smpB*(DE3) cells were used, they were continuously induced with 1uM IPTG). Cells were harvested and resuspended in 1mL of lysis buffer III [8M urea, 100mM potassium phosphate (pH8), 10mM Tris (pH8), and 5mM β-mercaptoethanol], and lysed by rocking at room temperature for 1 hr. Cell debris was collected by centrifugation and the supernatant was added to 100μL of Ni-NTA agarose resin (Qiagen). Resin binding was allowed to proceed for 1 hour at RT. Resin-sample slurries were then applied to a miniature chromatography column and allowed to flow through by gravity flow. The resin was washed 4 times with 1mL of lysis buffer III and his-tagged proteins were eluted in 200mL of an elution buffer containing: 8M urea, 100mM acetic acid, and 20mM β-Mercaptoethanol. Samples were resolved by electrophoresis on a 15% Tris-Tricine gel and Western blots were developed using either His-6-HRP (Santa Cruz)

or IR800-conjugated His-6 (Rockland) antibodies. Total level of endogenous tagging activity was quantified from Western blots using the IR dye conjugated antibody and the Odyssey Infrared Imaging System and data analysis software (LI-COR).

Gel Mobility Shift Assays

3'-end labeled tmRNA^{113} (~100pM) was incubated with varying concentrations of purified SmpB variants in a gel-shift buffer containing 50mM Tris (pH7.5), 10 mM $MgCl_2$, 300mM KCl, 2mM β-mercaptoethanol, 100mg/mL BSA, 0.01% NP-40 (v/v), 5% Glycerol(v/v), and 200nM total *E. coli* tRNA. Reactions were incubated for 60 min at 4ºC and then loaded onto 12% native polyacrylamide gels (run in 1/2X TBE (pH7.5)). Gels were dried and exposed to either X-ray film or phosphoimager screens. Phosphoimager data was collected using a Storm840 phosphorimager and analyzed with ImageQuant Tools (Molecular Diagnostics). The data analysis was performed according to Berggrun and Sauer (117). Briefly, I measured the fraction of the primary bound species at each SmpB concentration and determined the apparent equilibrium dissociation constant by curve fitting using the equation: $\Theta_{eq} = C/(1+K_d/[SmpB]_i)$, where Θ_{eq} is the fraction of RNA bound at equilibrium, C is a constant representing the maximum fraction bound of the specific bound species, and $[SmpB]_i$ is the initial concentration of SmpB. Note that $C < 1$ because of the occurrence of multiple bound species at SmpB concentrations where the fraction free is greater than zero.

Ribosome Association Assays

Ribosomes were purified from Δ*smpB*(DE3)/pETBA or pETBA^{H6} as previously described, with modifications (118). 750mL cultures were grown to OD_{600} ~ 1.0 under continuous low-level induction (1μM IPTG). Cells were harvested and resuspended in 4mL of buffer IIIA [10mM Tris (pH7.5), 100mM ammonium acetate, 10mM magnesium acetate, 6mM β-mercaptoethanol, and 100units/mL *RNase* inhibitor (Porcine, GE Healthcare)]. Cells were lysed by sonication, and the lysate was centrifuged at 30,000xg for 1hr. The S30 supernatant was then centrifuged at 100,000xg for 1hour to isolate crude ribosomes. Crude ribosome pellets were then washed with 1mL of Buffer IIIB [20mM Tris (pH7.5), 800mM ammonium acetate, 10mM magnesium acetate and 6mM β-mercaptoethanol], and resuspended in 1mL of buffer IIIA. Crude ribosomes were then layered onto 2mL of a 32% sucrose cushion containing: 32% sucrose, 20mM Tris (pH7.5), 500mM NH_4Cl, 10mM $MgCl_2$, 0.5mM EDTA, 6mM β-mercaptoethanol, and 100 units/mL *Rnase* Inhibitor. The sucrose cushions were then centrifuged at 100,000xg for 6hr. The ribosome pellets were again washed with 1mL of buffer IIIB and ribosomes were resuspended in 500μL of Buffer IIIA lacking *RNase* inhibitor.

For analysis of SmpB ribosome association, a fraction of this ribosome preparation was resolved on a 15% Tris-Tricine gel and Western blots were developed using either α-his6-HRP (Santa Cruz) or α-SmpB (a rabbit polyclonal antibody raised against *E.coli* SmpB) and α-rabbit HRP (Santa Cruz). For analysis of tmRNA ribosome association, the RNA from 250*μ*L of the ribosome preparation was extracted with 750 *μ*L of Tri Reagent LS (MRC). RNA samples were run on a 1.5% formamide-formaldehyde

denaturing agarose gel and Northern blots were developed using a biotinylated full-length SsrA dsDNA probe and a Biotin detection kit (Roche).

2.4 Results

SmpB C-Terminal tail mutants are defective in supporting the tagging function of *trans*-Translation *in vivo*

The C-terminal tail of SmpB contains several highly conserved and charged residues. I have chosen to focus on the residues D137, K138 and R139 that are 100%, 98% and 100% conserved respectively, as determined by analysis of 115 SmpB protein sequences. Single, double and triple alanine substitution mutations as well as charge altering mutations D137R, K138E, R139E and D137R/R139E were generated on plasmid pet28BA^{H6} via the Statagene quick change kit. The plasmid harbors the *smpB* gene and a fully functional tmRNA variant (tmRNA^{H6}) that encodes ANDEHHHHHH in place of the normal ANDENYALAA degradation tag. The altered mRNA sequence permits endogenously tagged proteins to be purified by Ni^{2+} -NTA chromatography and detected by Western blot analysis.

I transformed an *smpB* deletion strain (Δ*SmpB*) with the pET28BA^{H6} plasmid harboring various SmpB substitution mutants and purified endogenous His-6 tagged substrates of the SmpB-tmRNA system. SmpB single-alanine mutations of residues D137, K138, and R139 had a modest affecton tagging ability (Data Not Shown).

Interestingly, the mutants K138A/R139A, D137A/K138A/R139A and R139E were unable to support the protein tagging function of *trans*-translation. The substitution mutant R139E and the double alanine mutant, K138A/R139A both displayed a greater than 50% loss of tagging ability. The triple-alanine mutant showed the greatest loss of tagging ability, greater than 85% as compared to wild-type SmpB (Figure 2.1 lanes 5,8,9). Remarkably, the R139E tagging phenotype was rescued by replacing D137 with arginine (D137R/R139E) (Figure 2.1 compare lanes 5 & 6). Analysis of the Coomassie stained gels revealed that the decrease in tagging was not due to a decrease in the amount of protein loaded per lane (data not shown). Likewise, the decrease in endogenous tagging was not due to a decrease in the level of expression of each SmpB variant analyzed (data not shown). It is clear that I have identified SmpB amino acid residues that are critical for the SmpB-tmRNA protein tagging function of *trans*-translation.

SmpB C-Terminal tail mutants stably bind tmRNA *in vitro*

I further characterized these mutants by testing their ability to bind tmRNA *in vitro*. The SmpB variants were expressed and purified to homogeneity by Ni-NTA chromatography followed by ion exchange chromatography. The equilibrium dissociation constants (K_d) of each protein for interaction with tmRNA variant ($tmRNA^{113}$), which contains the tRNA-like domain of tmRNA and a section of the stem region just beyond the tRNA-like domain, were determined using a quantitative electrophoretic mobility shift assay (EMSA). The EMSA assays were done in 300mM KCl in the presence of a 100 fold molar excess of total *E. coli* tRNA to eliminate

nonspecific binding. Analysis of my data clearly shows that the SmpB mutants bind tmRNA *in vitro* with high affinity and specificity with equilibrium dissociation constants (Kd) similar to $SmpB^{WT}$ (Table 2.1).

SmpB C-Terminal tail mutants stably associate with ribosomes *in vivo*

Having established that the highly conserved amino acids D137, K138, and R139 play a key role in *trans*-translation, I wished to ascertain whether the decrease in endogenous tagging activity was due to a compromised ability of the mutants to bind tmRNA or to recognize stalled ribosomes. tmRNA binding and ribosome association are required for *trans*-translation, and a defect in either function could lead to a decrease in endogenous tagging activity.

The SmpB mutants were assayed *in vivo* for their ability to associate with ribosomes and facilitate the stable association of tmRNA with ribosomes. Purification of 70S ribosomes by density gradient ultracentrifugation, and detection of SmpB and tmRNA by Western and Northern blotting, clearly shows that these SmpB variants are fully proficient in associating with ribosomes (Figure 2.2A). Furthermore, they also facilitate the stable association of tmRNA with ribosomes (Figure 2.2B). Therefore, the C-terminal tail mutations do not affect the ability of SmpB to associate with ribosomes nor do they affect the delivery of tmRNA to those ribosomes.

Overall, these SmpB variants support all of the currently known functions of SmpB protein binding to tmRNA and facilitating its delivery to stalled ribosomes. From analysis of the *trans*-translation mechanism, it is clear that SmpB must play a role in

trans-translation after association with 70S ribosomes but prior to addition of the tmRNA-encoded peptide tag. Interestingly, deletion of the C-terminal tail leads to a similar phenotype, association of the SmpB-tmRNA complex with ribosomes but no addition of the tmRNA-encoded peptide tag(64). It has been shown by our lab and others that the addition of the tmRNA-charged alanine to the nascent peptide does not occur with SmpB C-terminal truncation mutants (63, 119). Therefore, the defect in the C-terminal tail mutants most likely affects accommodation of tmRNA into the A-site in the absence of codon-anticodon interactions. The various SmpB C-terminal tail mutants will be useful tools in determining what role the SmpB C-terminal tail plays in this process.

2.5 Discussion

SmpB protein is highly conserved throughout Eubacteria and contains a number of highly conserved and invariant residues. I have demonstrated that the highly conserved SmpB C-terminal tail residues D137, K138 and R139 are critical for a novel function of SmpB in *trans*-translation. I have shown that mutations of these highly conserved residues leads to a marked decrease in the level of endogenously tagged proteins *in vivo*. The most severe phenotype was seen with $SmpB^{D137A/K138A/R139A}$ leading to a greater than 85% decrease in the ability to tag endogenous proteins as compared to $SmpB^{WT}$ (Figure 2.1). Interestingly though, mutations to these residues does not affect the ability of SmpB to bind tmRNA *in vitro* (Table 2.1). Furthermore, mutation of these highly conserved residues does not impair the ability of SmpB to associate with 70S ribosomes (Figure 2.2A). Similarly, these mutations do not affect the ability of tmRNA

to associate with 70S ribosomes (Figure 2.2B). Mutation of these SmpB residues leads to a phenotype similar to SmpB C-terminal tail deletion mutants, inability to support the tmRNA tagging function but full competence in tmRNA binding and ribosome association(64). Information gleaned from the SmpB C-terminal tail deletion mutants indicates that the tmRNA alanine charge is not appended to the incomplete peptide bound to the ribosome(64). Taken together, this indicates that the novel role of SmpB on the ribosome is post-ribosome association but prior to the addition of the tmRNA alanine charge. It is therefore likely that the critical role played by the SmpB residues D137, K138 and R139 is post ribosome association and prior to the addition of the tmRNA alanine addition.

During translation of an mRNA, a ternary complex containing the cognate tRNA and EF-Tu(GTP) recognizes the appropriate codon in the ribosomal A-site(1-3, 13, 120, 121). Upon binding of the ternary complex, proper codon-anticodon interactions leads to hydrolysis of GTP by EF-Tu and subsequent release of EF-Tu, allowing full accommodation of the tRNA in the A-site and positioning of the acceptor stem in the peptidyl transferase center(1, 2, 15, 16). tmRNA lacks an anticodon loop, and the ribosomal A-site is necessarily empty when recognized by the tmRNA-SmpB complex. Therefore, interactions of the ternary complex containing SmpB-tmRNA and EF-Tu(GTP) leading to GTP hydrolysis must be unique to the *trans*-translation mechanism. The interactions that facilitate GTP hydrolysis, of EF-Tu bound to tmRNA are currently unknown. However, Karzai et al. originally proposed that SmpB may be playing a role as an anticodon mimic, similar to the role played by domain IV of EF-G and stimulating activation of the GTPase domain of EF-Tu(121, 122). If the C-terminal tail of SmpB is

acting as an anticodon mimic and stimulating GTP hydrolysis the D137, K138 and R139 residues are possibly playing a key role, likely by interacting with rRNA within the ribosomal A-site. Interestingly, some groups report that modeling of the tmRNA-SmpB complex onto 70S ribosomes places the C-terminal tail in the direction of the ribosomal A-site and towards ribosomal nucleotides critical for detecting traditional codon-anticodon interactions(41). It is possible that the SmpB mutants in this region of the C-terminal tail and specifically the charge variant, $SmpB^{R139E}$, might interfere with the ability of the SmpB C-terminal tail to form a structure within the context of the ribosome. The ability of the SmpB C-terminal tail to form a structure in this region might be necessary for proper accommodation of the SmpB-tmRNA complex into the ribosomal A-site. This suggests that interactions of the Asp-137, Lys-138 and Arg-139 are necessary for allowing formation of this structure. This also suggests that the $SmpB^{D137R/R139E}$ mutant is able to make the requisite contacts within the context of the ribosome necessary for formation of this structure. This might indicate that the Asp-137, Lys-138 and Arg-139 are involved in formation of stabilizing salt bridges that allow formation of this structure.

I have demonstrated that conserved amino acids within the C-terminal tail of SmpB are critical in the protein tagging function in *trans*-translation. The role of these amino acids in the mechanism of *trans*-translation is post tmRNA-SmpB association as well as post SmpB-tmRNA ribosome association. I propose that these amino acids are involved in interactions on the ribosome that elicit GTP hydrolysis by EF-Tu and allow trans-peptidation of the tmRNA alanine charge allowing proper accommodation of the SmpB-tmRNA complex into the ribosomal A-site.

2.6 Figures

Figure 2.1

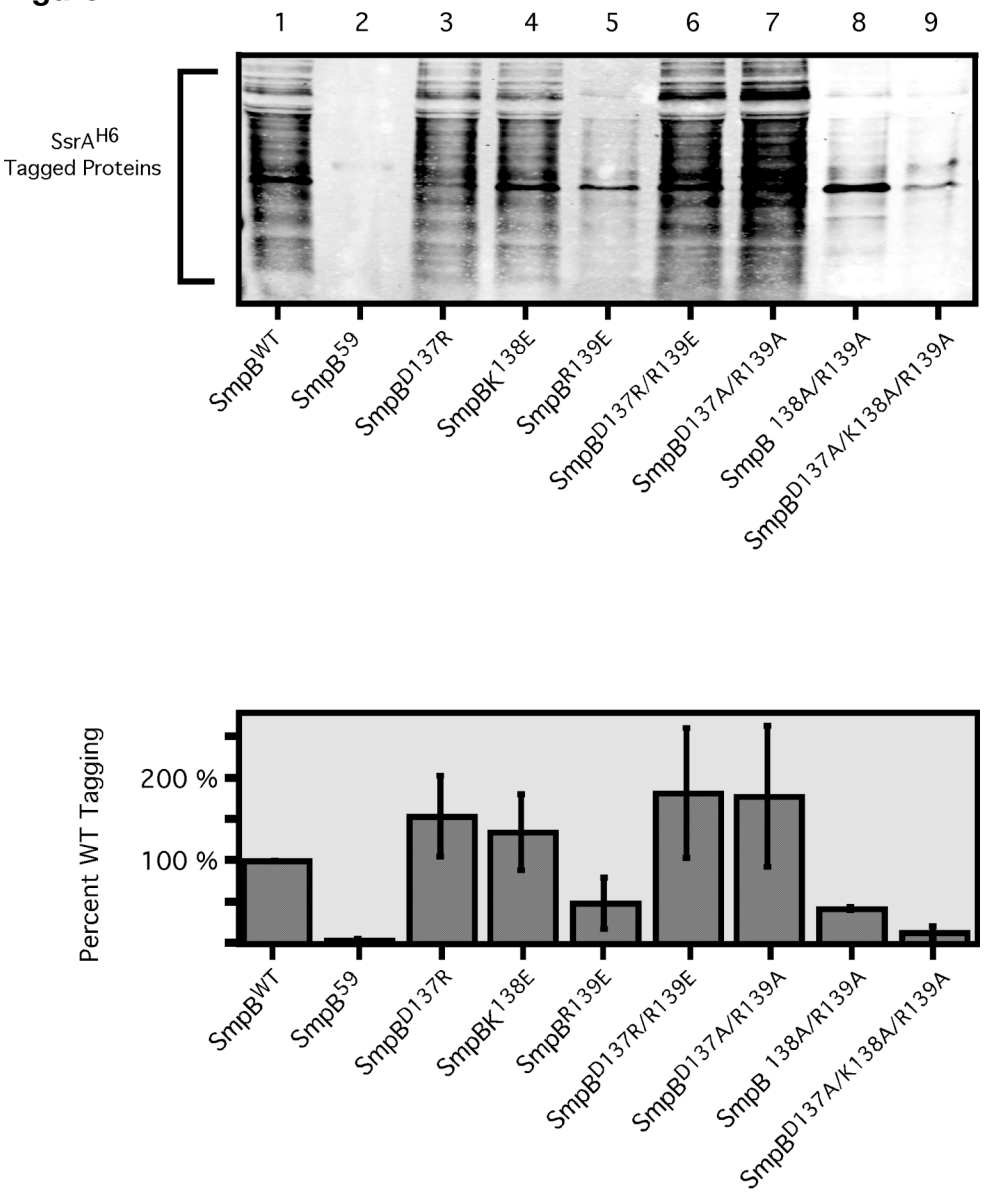


Figure 2.1: Analysis of the endogenous tagging activity of E. coli SmpB alanine substitution mutants. (A) A representative Western blot, developed with antibodies against the His-6 epitope, displaying the endogenously tagged proteins. Lane numbers are depicted on top (B) Bar graphs, representing the mean and standard deviation of 3 independent tagging assays, display the level of tagging activity as compared to wild-type SmpB protein. Wild-type SmpB is used as a positive control and $SmpB^{59}$, a nonfunctional truncated SmpB variant, is used as a negative control.

Figure 2.2

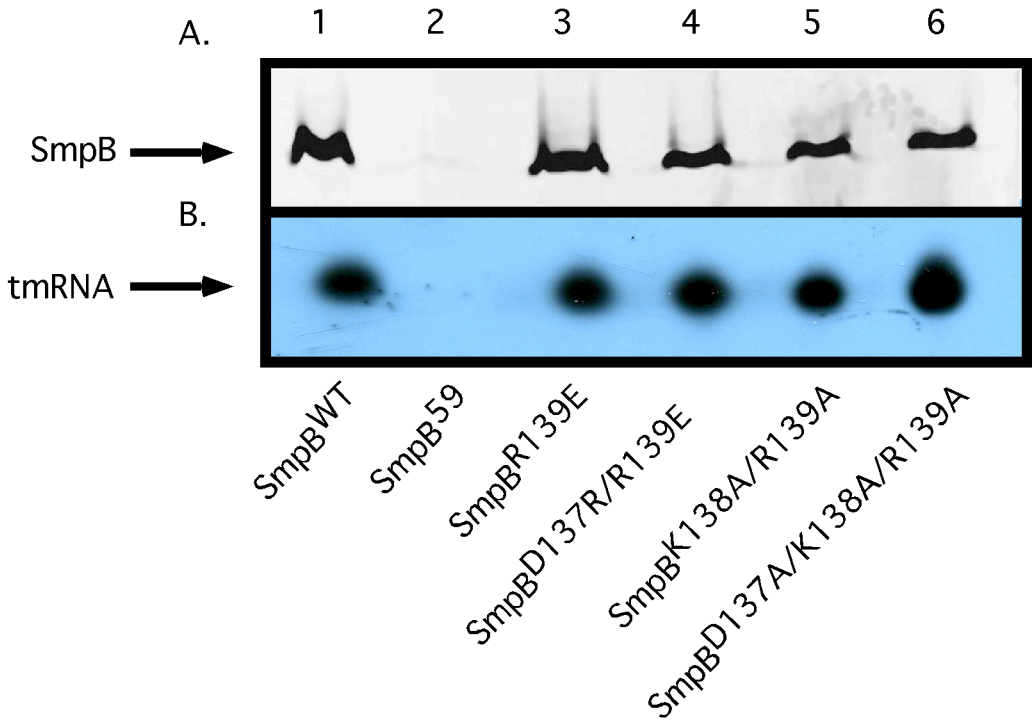


Figure 2.2: Ribosome association assays. (A) Western blot analysis with anti-SmpB antibodies displaying the amount of wild-type and select mutant SmpB protein associated with 70S ribosomes *in vivo*. (B) Northern blot analysis with a tmRNA specific probe to detect tmRNA associated with 70S ribosomes.

Table 2.1

tmRNA binding parameters, derived from curve-fitted gel mobility-shift data, for wild-type *E. coli* SmpB protein and SmpB C-terminal variants in the presence of a 100 fold molar excess of total *E. coli* tRNA.

E. coli *SmpB*	Kd (M)
$SmpB^{WT}$	$1.17 \times 10^{-9} \pm 1 \times 10^{-9}$
$SmpB^{R139E}$	$0.411 \times 10^{-9} \pm 2 \times 10^{-9}$
$SmpB^{K138A/R139A}$	$0.877 \times 10^{-9} \pm 2 \times 10^{-9}$
$SmpB^{D137A/K138A/R139A}$	$0.688 \times 10^{-9} \pm 3 \times 10^{-9}$

Chapter 3: The Role of Conserved Surface Amino Acids in Binding of SmpB Protein to tmRNA

*** The work contained within this chapter was published in The Journal of Biological Chemistry in 2006(54).**

3.1 Summary

Bacteria possess a unique salvage mechanism for rescuing ribosomes stalled on aberrant mRNAs. This salvage mechanism, termed *trans*-translation, is mediated by the versatile tmRNA (alternatively known as SsrA and 10Sa RNA) and Small Protein B (SmpB), its binding partner. The specific and direct binding of SmpB to tmRNA has been shown to facilitate its delivery to stalled ribosomes. Once properly positioned in the ribosomal A-site, tmRNA acts as a tRNA and an mRNA to mediate the co-translational addition of a short peptide tag to the C-termini of nascent polypeptide chains, marking them for degradation by C-terminal specific proteases. I have used systematic alanine scanning mutagenesis combined with *in vivo* and *in vitro* assays to investigate the functional significance of a number of highly conserved residues of the SmpB protein. I present *in vivo* evidence to demonstrate that amino acid residues E31, L91, and K124, which are clustered along an exposed surface of the protein, play a crucial role in the tmRNA-mediated nascent-peptide tagging process. My analysis suggests that the peptide-tagging defect exhibited by the SmpB substitution variants is due to their inability to facilitate the delivery of tmRNA to stalled ribosomes. Moreover, I present evidence to

demonstrate that the ribosome-association defect of these SmpB variants is due to their reduced binding affinity for tmRNA. Consistent with these findings, I present biochemical evidence to directly demonstrate that residues E31, L91, and K124 are essential for the tmRNA binding activity of SmpB protein.

I have also investigated the interactions of SmpB-tmRNA orthologues from the thermophilic bacterium *T. tengcongensis* (Tten). I have substituted the equivalent Tten-SmpB surface residues and determined the role of these residues in SmpB-tmRNA binding. I demonstrate that residues E28, L87, H89 and K120 make significant energetic contributions to the binding of Tten SmpB protein to Tten tmRNA. Taken together, these results demonstrate the functional significance of residues E31, L91, and K124 in SmpB binding to tmRNA, suggesting that this cluster of evolutionarily conserved residues might represent a core contact surface for recognition of tmRNA.

3.2 Introduction

tmRNA (also known as SsrA and 10Sa RNA) is a small, highly structured RNA that is found in all bacteria. tmRNA possesses a tRNA-like domain and an mRNA-like segment that jointly endow it with both tRNA- and mRNA-like functions(52, 93, 123-129). The model for tmRNA function, also known as *trans*-translation, proposes that alanine-charged tmRNA (in a quarternary complex with SmpB, EF-Tu, and GTP) recognizes stalled ribosomes, binds at the ribosomal A-site, and donates its alanine charge to the growing polypeptide(65, 130, 131). The ribosomal reading frame then switches to the mRNA portion of tmRNA, adding a degradation tag to the C-terminus of

targeted polypeptides. tmRNA-tagged proteins are then recognized by cellular proteases and efficiently degraded(52, 93, 126-128).

Small protein B (SmpB) is essential for all known activities of tmRNA; however, its specific function in *trans*-translation is less well explored. The known functions of SmpB are specific binding to tmRNA and promoting stable association and engagement of the SmpB•tmRNA complex with stalled ribosomes(132). Recently, a great deal of structural information regarding SmpB and tmRNA has become available. NMR solution structures of SmpB from *Aquifex aeolicus* and *Thermus thermophilus* along with a co-crystal structure of *Aquifex aeolicus* SmpB in complex with the tRNA-like domain of tmRNA have been solved(40, 59, 133). The core SmpB structure is quite similar in all structural models.

In studies described here, I have sought to gain a better understanding of the role and energetic contributions of highly conserved surface amino acids to the interaction of SmpB protein with tmRNA. Clustal X (134, 135) alignment of SmpB protein orthologs shows the presence of several conserved polar and hydrophobic residues that contribute to the identity observed among different prokaryotic SmpB proteins. I have focused on a number of conserved amino acid residues closely clustered on one surface of the SmpB protein (Figure 3.1). I provide *in vivo* and *in vitro* evidence for the participation of these amino acids in *trans*-translation, ribosome association and tmRNA binding. More specifically, I show that single and double alanine substitutions at amino acid residues E31, L91, and K124, within the oligonucleotide binding-fold (OB-fold), of *E. coli* SmpB cause a decrease in the *in vivo* tagging propensity of the SmpB protein. Additionally, I demonstrate that the mutants are deficient in their ability to deliver tmRNA to 70S

ribosomes. Finally, I show that SmpB residues E31, L91, N93, K124 are directly involved in interaction with tmRNA. Single alanine substitutions of each one of these amino acid residues result in a significant decrease in the binding affinity of SmpB protein for tmRNA *in vitro*. The decrease in binding affinity of the SmpB variants for tmRNA ultimately leads to a reduction in the ability of SmpB protein to effectively deliver tmRNA to stalled ribosomes and tag endogenous protein substrates.

Moreover, I have probed the SmpB-tmRNA complex of the thermophilic bacterium, *Thermoanerobacter tengcongenesis* (Tten), and demonstrate that the equivalent residues (E28, H89, L91, and K120) make significant energetic contributions to the binding of Tten SmpB protein to Tten tmRNA. Taken together, these results are consistent with my findings for the *E. coli* SmpB-tmRNA interaction and suggest that these clustered surface residues are conserved in Eubacteria primarily to maintain the critical contacts in binding of SmpB protein to tmRNA.

3.3 Experimental Procedures

Analysis of Endogenous tmRNA-H6 Mediated Tagging

All single and double SmpB mutations were introduced by PCR mutagenesis using the Stratagene Quick Change Kit. Plasmid pET28 $BA^{His\text{-}6}$ (53, 57) for $tmRNA^{H6}$, tmRNA RNA with a modified mRNA-like domain coding for a six-histidine tag. *E. coli* strain W3110 Δ*SmpB*/(DE3) was transformed with individual pET28 BA^{H6} plasmid variants containing $SmpB^{WT}$ or one of the SmpB alanine mutants, $SmpB^{E31A}$, $SmpB^{L91A}$,

$SmpB^{K124A}$, $SmpB^{N93A}$, $SmpB^{Q94A}$, $SmpB^{E31A/L91A}$, $SmpB^{E31A/K124A}$, $SmpB^{L91A/K124A}$, $SmpB^{N93A/Q94A}$, $SmpB^{E31A/L91A/K124A}$. Tagging-assay cultures were grown in 50 ml Luria Broth, containing 50 ug/ml Kanamycin and 3 μM isopropyl β-D-thiogalactoside (IPTG), to $O.D._{600}$ of 0.85. Cultures were harvested, resuspended in lysis buffer (8M Urea, 1% Triton X-100, 100 mM NaH_2PO_4, 10 mM Tris pH8.0) and lysed by shaking at RT for 1 hr. Cell debris was removed by centrifugation for 30 min at 30,000xg and the supernatant was mixed with 0.1 ml Ni^{2+}-NTA slurry (Qiagen), pre-equilibrated in lysis buffer, in a micro bio spin chromatography column (BioRad). Binding reactions were incubated at 4°C for 30 minutes with continuous rocking. The column was then centrifuged for 1 min in a tabletop centrifuge at 4000 rpm to pellet the Ni^{2+}-NTA beads, washed three times with 1 ml of lysis buffer and the bound proteins were eluted with 0.150 ml of elution buffer (8 M Urea, 1% Triton X-100, 10 mM βME, 100mM acetic acid). The pH was raised by adding 1:10 (vol:vol) of 2 M Tris pH 9.5. A fraction of the eluate (100 μL) was resolved by electrophoresis on a 15% Tris-Tricine Gel. Proteins were transferred to a nitrocellulose membrane (0.05 μC BioRad) and Western blots were developed with His probe H-15 antibodies (Santa Cruz) and an IR 680 nm dye-conjugated secondary antibody (Molecular Probes). Membranes were scanned on an Odyssey scanner (LI-COR) and quantified with the Odyssey Software package. Each assay was repeated at least three times and the total signal in each lane of the tagging assay was quantified and compared to that of $SmpB^{WT}$ to generate % of wild type activity.

Ribosome Association Assay

For 70S ribosome preparations, 750 ml of W3110 *ΔsmpB*/(DE3) containing plasmid pET28 BA^{wt} or pET28 BA with specified SmpB amino acid substitutions were grown in LB containing 3 μM IPTG to $O.D._{600}$ of 0.8-1.0. Bacterial cells were harvested, washed in 50 mM Tris (pH 7.5), harvested by centrifugation and stored at -80°C. Cell pellets were resuspended in Buffer A (20 mM Tris pH7.5, 300 mM NH_4Cl, 10 mM $MgCl_2$, 0.5 mM EDTA, 6 mM β-mercaptoethanol, 10 U/ml SuperASE In (Ambion) and lysed by gentle sonication. Lysates were centrifuged at 33,000xg for 30 minutes. Supernatants were transferred to new tubes and centrifuged again at 33,000xg for 30 minute. Typically, a 19 ml aliquot of the supernatant was layered onto a 32% sucrose cushion in buffer B (20 mM Tris (pH7.5), 500 mM NH_4CL, 10 mM $MgCl_2$, 0.5 mM EDTA, 6 mM β-mercaptoethanol, 10U/ml SuperASE In (Ambion)) and centrifuged at 85,000xg for 22 hrs. The pellet containing tight-coupled ribosomes was washed twice with 5ml of cold buffer B. Pellets were resuspended in 0.25 ml buffer A and equivalent numbers of ribosomes were loaded onto a 10-50% sucrose cushion in buffer A. The sucrose gradients were centrifuged at 42,000xg for 17 hrs. Gradients were fractionated and the fractions corresponding to purified 70S ribosomes were pooled and used for Western and Northern blot analysis. RNA for Northern blot analysis was extracted with Tri-LS reagent (Molecular Research) and equal amounts of RNA were loaded onto 1% formaldehyde agarose gels, transferred to Hi-Blot Nylon membrane (Amersham) and probed with a psoralen-biotin (Ambion) labeled full length tmRNA probe. For Western blot analysis, an equal number of ribosomes were loaded per lane, and the associated

proteins were resolved by electrophoresis on 15% Tris-Tricine gels. Western blots were developed with antibodies raised against purified SmpB protein and a secondary IR 800nm dye-conjugated secondary antibody (Molecular Probes).

Purification of SmpB protein Variants

Bacterial strain BL21 (DE3)/plysS (Stratagene) was transformed with plasmid pET28 BA^{H6} harboring $SmpB^{WT}$ or one of the alanine mutants: $SmpB^{E31A}$, $SmpB^{L91A}$, $SmpB^{K124A}$, $SmpB^{N93A}$, $SmpB^{Q94A}$, $SmpB^{E31A/L91A}$, $SmpB^{E31A/K124A}$, $SmpB^{L91A/K124A}$, $SmpB^{N93A/Q94A}$, $SmpB^{TtenWT}$, $SmpB^{TteneE28A}$, $SmpB^{TtenL87A}$, $SmpB^{TtenH89A}$, $SmpB^{TtenR90A}$ or $SmpB^{TtenK120A}$. Typically, cells were grown in three liters of LB at 37° to an $O.D._{600}$ of ~0.5 and induced with 1 mM IPTG for 3 hr. Cells were harvested and resuspended in lysis buffer (1 M NH_4Cl, 150 mM KCl, 50 mM Tris (pH 8.0), 2 mM β-mercaptoethanol, 20 mM immidazole) and lysed by sonication (3x30 sec pulses, with the addition of 0.1 ml of 0.1M PMSF after each pulse). Lysates were centrifuged for 1 hr at 13,000 rpm in an SS-34 rotor. Due to the greater thermal stability of the Tten SmpB protein, an additional heat treatment step was included for wild type Tten SmpB and all of its alanine substitution variants. The S30 supernatants of Tten SmpB proteins were heated at 65°C for 10 min. This treatment results in the denaturation and precipitation of greater than 80% of soluble *E. coli* proteins, whereas the Tten SmpB variants are entirely unaffected and remain soluble. The heat-treated protein samples were centrifuged for 1 hr at 15,000 rpm to pellet the denatured *E. coli* proteins. The supernatants were mixed with 2 ml Ni^{2+}-NTA resin (Quiagen) pre-equilibrated in lysis buffer and rocked for 1 hr at 4°C. The

Ni^{2+}-NTA resin was applied to a chromatography column and washed 3X with 30 ml lysis buffer. Proteins were eluted three times with 2 ml of elution buffer (150 mM KCl, 50 mM Tris (pH8.0), 200 mM Immidazole, 20 mM β-mercaptoethanol). The eluate was then diluted in FPLC buffer A (50 mM KCl, 50 mM Hepes (pH 7.5), 5 mM $MgCl_2$, 2 mM β-mercaptoethanol) and loaded onto a MonoS ion-exchange column (Amersham-GE-Healthcare). A gradient of 50 mM KCl to 850 mM KCl was developed over 20 column volumes to isolate the SmpB protein. SmpB protein, with greater than 95% purity, elutes at approximately 500 mM KCl under these conditions. Protein concentrations were determined by absorbance at 280 nM, using SmpB extinction coefficient of 29575 M^{-1} cm^{-1} for *E. coli* SmpB and its variants, and 10430 M^{-1} cm^{-1} for Tten SmpB and its variants. Protein aliquots were stored at -80°C until needed.

Electrophoretic Mobility Shift Assays

All tmRNA variants were produced as described previously(64). The *csrB* gene was PCR amplified from *E. coli* genomic DNA using a 5'-primer (5'-CGAATTCTAATACGACTCACTATAGGTGTCTTCAGGACGAAGAAC-3') and a 3'-primer (5'-AAAAGGGGTACTGTTTTACCAG-3'). The amplified product was gel purified and reamplified to incorporate a T7 promoter sequence at its 5'-end. CsrB RNA was transcribed using T7 RNA polymerase (USB) in accord with the manufacturers recommendations. Template DNAs were digested with DNase I and the transcription products were phenol/chloroform extracted and purified by electrophoresis on denaturing polyacrylamide agarose gels. tmRNA variants used in this study were labeled at their 3'-

end as described by Sundermeier et al (64). Electrophoretic mobility shift assays (EMSA) were performed essentially as described(64), with minor modifications. Briefly, *E. coli* and *Tten* SmpB protein mutants were diluted to the desired protein concentrations in EMSA buffer (50 mM Tris (pH 7.5), 2 mM MgCl2, 300 mM KCl, 100 μg/ml bovine serum albumin, 2 mM β-mercaptoethanol 0.01% NP-40 (v/v), 10% glycerol (v/v)) in the presence of 100 nM CsrA RNA as a nonspecific competitor. Approximately 100 fmol of 3' end labeled tmRNA^{113} RNA (~1000 cpm per reaction) were added to each tube and incubated at room temperature for 30 minutes. Samples were loaded on a 12% non-denaturing gel and resolved by electrophoresis at 200V in 0.5X TBE to separate the free RNA from RNA-protein complexes. Gels were run at 4°C, dried and exposed overnight to phosphoimager screens. All binding experiments for *E. coli* and *T. tengcongenesis* SmpB mutants, were performed at least in triplicate, covering a protein concentration range of 1nM to1.5μM. Data analysis was performed according to Berggrun and Sauer . Briefly, the fraction of the primary bound species at each SmpB concentration was determined, and the apparent equilibrium dissociation constant was obtained by curve fitting using the equation: $\Theta_{eq} = C/(1+K_d/[SmpB]_i)$, where Θ_{eq} is the fraction of RNA bound at equilibrium, C is a constant representing the maximum fraction bound of the specific bound species, and $[SmpB]_i$ is the initial concentration of SmpB. ΔG values were calculated from the relationship $\Delta G = -RT \ln (K_d)$, where R is the universal gas constant (1.9872 calories mol^{-1} K^{-1}) and T is the temperature in Kelvin ($T_{Kelvein} = T_{celsius} + 273$).

3.4 Results

Mutations in conserved SmpB amino acids decrease the level of *in vivo* tagged proteins

The SmpB protein is a requisite component of the *trans*-translation process. It performs several key functions, including specific binding to tmRNA, recognition of stalled ribosomes and proper positioning of tmRNA in the ribosomal A-site (93, 128, 136, 137). Sequence alignment analysis of SmpB protein reveals the identity of a number of invariant and semi-invariant amino acid residues that are conserved across many diverse bacterial species(83). To gain insights into the functional significance of these highly conserved amino acid residues, I carried out a systematic alanine-scan mutagenesis of strategic amino acid residues of the *E. coli* SmpB protein and evaluated their contributions to known SmpB functions in *trans*-translation. I deemed alanine substitutions ideal, as they eliminate the side chain, beyond the β-carbon, without altering the main-chain conformation or imposing steric or electrostatic restrictions. I have previously reported on the functional significance of a number of highly conserved amino acid residues located in the C-terminal tail of the protein(119). Here, I focused specifically on several highly conserved amino residues (E31, L91, and K124) and two variably conserved residues (N93, Q94) that are clustered along one exposed surface of the protein (Figure 3.1A). The mutants were generated by site directed mutagenesis using pET28BA^{H6}, a plasmid that harbors the *smpB* gene and a fully functional *ssrA* variant

(tmRNA^{H6}) that encodes for ANDEHHHHHH in place of the normal ANDENYALAA degradation tag. The altered mRNA sequence permits endogenously tagged proteins to be purified by Ni^{2+} -NTA chromatography and detected by Western blot analysis.

To probe the contributions of individual amino acids to known SmpB functions, I evaluated the effect of single alanine substitutions on the SmpB-tmRNA mediated trans-translation process *in vivo*. To this end, I transformed an *smpB* deletion strain (ΔSmpB) with the pET28BA^{H6} plasmid harboring single alanine substitution mutants, of SmpB, and purified endogenous His-6 tagged substrates of the SmpB-tmRNA system. The purified proteins were resolved by electrophoresis on SDS-PAGE, and the levels of endogenously tagged proteins were determined by Western-blot analysis (Figure 3.2A). This analysis revealed that individual alanine substitutions of the two variably conserved SmpB residues (N93 and Q94) did not have a substantial effect on the ability of tmRNA to tag endogenous substrates. In contrast, alanine substitutions of SmpB residues E31, L91, and K124 resulted in a modest and reproducible decrease in the level of endogenously tagged proteins. Compared to the wild-type SmpB protein, these mutants consistently displayed a 15 to 20% reduction in endogenous tagging activity (Figure 3.2 A, C). Based on the modest decrease in endogenous tagging activity observed with the single amino acid substitution mutants and to gain further insight into the contributions of these residues, I generated a number of double alanine substitution mutants of SmpB protein, including E31A/L91A (SmpB^{EL}), E31A/K124A (SmpB^{EK}), L91A/K124A (SmpB^{LK}), and N93A/Q94A (SmpB^{NQ}).

Evaluation of the ability of the double alanine substitution mutant SmpB^{NQ} to support *trans*-translation *in vivo* revealed little or no loss of tagging activity. In contrast,

double alanine substituted mutants $SmpB^{EL}$, $SmpB^{LK}$, and $SmpB^{EK}$ showed a marked decrease in endogenous tagging activity (Figure 3.2A, lanes 3-5). Compared to wild type SmpB, the tagging propensity of these variants was reduced by 30%, 50% and 65%, respectively (Figure 3.2C). A triple alanine substituted mutant, E31A/L91A/K124A ($SmpB^{ELK}$), showed a maximum of 70% loss of *in vivo* tagging capacity (Figure 3.2C).

The decrease in endogenous tagging capacity of these mutants correlates well with the number of amino acids mutated along the OB-fold, and with the degree of conservation of the mutated amino acids. More specifically, amino acids along β sheets β2(E31), β5(L91), and β7(K124), which are 93%, 100% and 99% conserved, when mutated to alanine show the greatest loss in tagging propensity.

Substitution of *E. coli* SmpB residues E31, L91, K124 decreases tmRNA association with 70S ribosomes *in vivo*

Having established that the highly conserved amino acid E31, L91 and K124 play a role in *trans*-translation, and knowing that these amino acid residues are clustered along a distinct surface of the SmpB protein, I wished to ascertain whether the decrease in endogenous tagging activity was due to a compromised ability of the mutants to bind tmRNA or to recognize stalled ribosomes. Both tmRNA binding and ribosome association are required for *trans*-translation and a defect in either could lead to a decrease in endogenous tagging activity(119, 136). To appraise ribosome association propensities of the mutants, I purified 70S ribosomes from *ΔsmpB* cells harboring wild-type SmpB, or one of its variants ($SmpB^{EL}$, $SmpB^{EK}$, $SmpB^{LK}$, and $SmpB^{ELK}$), and probed

for the presence of SmpB protein and tmRNA. Western-blot analysis of 70S ribosome-associated SmpB protein showed that all of the double and triple alanine substituted SmpB mutants had substantially decreased levels of SmpB protein associated with 70S ribosomes (Figure 3.3A, lanes 4,6). The most notable decrease was observed with double alanine mutant $SmpB^{EK}$ and the triple alanine mutant $SmpB^{ELK}$. Further analysis of the levels of each protein showed that the ribosome association defects were not due to a decrease in the total amount of SmpB present in the cells, as similar amounts of all SmpB mutants were present in the S30 extract from these cells (Figure 3.4).

Since SmpB function is required for stable association of tmRNA with stalled ribosomes, I therefore analyzed the level of tmRNA associated with 70S ribosomes. Consistent with the above observations, the level of 70S ribosome tmRNA associated was considerably diminished in all SmpB mutants tested (Figure 3.3B). The greatest decrease in the level of ribosome associated tmRNA was observed with $SmpB^{ELK}$, followed by $SmpB^{EK}$, $SmpB^{LK}$, and $SmpB^{EL}$ (Figure 3.3B). Taken together these data suggest that the ribosome association defects of the SmpB mutants and the dramatic decrease in the levels of ribosome associated tmRNA are most likely the cause of the observed endogenous tagging defects (Figure 3.2).

E. coli SmpB single amino acid substitution mutants are defective in binding tmRNA *in vitro*

A critical first step in *trans*-translation is the binding of SmpB protein to tmRNA.

Formation of a stable SmpB-tmRNA complex is critical for the facilitated delivery of tmRNA to stalled ribosomes. Thus, the ability of SmpB to deliver tmRNA to the ribosome is directly related to the binding affinity and specificity of SmpB protein for tmRNA. In order to characterize this step in *trans*-translation, I generated expression constructs of single and double alanine substitution mutants of key amino acid residues along the OB-fold of SmpB protein. Expression and purification of these mutant proteins followed the same basic procedure as for wild-type SmpB protein (see Materials and Methods). In all cases, the mutant proteins were purified to homogeneity as detected by Coomassie-stained SDS-PAGE gel electrophoresis.

The equilibrium dissociation constants (K_d) of each protein for interaction with a tmRNA variant ($tmRNA^{113}$), which contains the tRNA-like domain of tmRNA and a section of the stem region just beyond the tRNA-like domain, were determined using electrophoretic mobility shift assay (See Materials and Methods). All binding assays were performed at least in triplicate and K_d values were derived by curve fitting using the equation: $\Theta_{eq} = C/(1+K_d/[SmpB]_i)$, where Θ_{eq} is the fraction of RNA bound at equilibrium, C is a constant representing the maximum fraction bound of the specific bound species, and $[SmpB]_i$ is the initial concentration of SmpB.

The single alanine substitution mutants increase the equilibrium dissociation constants from 4 nM for wild-type SmpB protein to 26 nM for N93A, 29 nM for E31A, 49 nM for K124A, and 58 nM for L91A (see Table 3.1). Compared to wild-type SmpB, the N93A mutation reduces binding ~6-fold ($\Delta\Delta G$ = 1.0 kcal/mol), the E31A mutation reduces binding ~7-fold ($\Delta\Delta G$ = 1.2 kcal/mol), the K124A mutation reduces binding ~12-fold ($\Delta\Delta G$ = 1.5 kcal/mol), and the L91A mutation reduces binding ~14-fold ($\Delta\Delta G$ = 1.6

kcal/mol). Analysis of the single alanine SmpB variants revealed that amino acids E31, L91, N93 and K124 contribute significantly to the binding of SmpB to tmRNA (Table 3.1). Amino acid residue Q94 does not contribute significantly to the binding between SmpB and tmRNA RNA, making a marginal energetic contribution ($\Delta\Delta G$ = 0.3 kcal/mol) to complex formation. Clearly, each mutant binds to tmRNA with significantly lower affinity than wild-type SmpB, as expected if these highly conserved residues in the wild-type protein make important contacts with tmRNA.

Figure 3.5 shows the representative binding data and the fitted curves for $SmpB^{WT}$, $SmpB^{EL}$, and $SmpB^{EK}$. When these single amino acid mutations are combined, the loss of multiple specific contacts leads to a further decrease in binding affinity. The greatest loss of binding affinity was observed with $SmpB^{ELK}$, $SmpB^{EK}$ and $SmpB^{LK}$, which have K_d values of 174nM, 138 nM and 87 nM, respectively. Compared to wild-type SmpB, the E31A/L91A mutations reduce binding ~8-fold ($\Delta\Delta G$ = 1.2 kcal/mol), the E31A/K124A mutations reduce binding ~35-fold ($\Delta\Delta G$ = 2.1 kcal/mol), the L91A/K124A mutations reduce binding ~22-fold ($\Delta\Delta G$ = 1.8 kcal/mol) and the E31A/L91A/K124A mutations reduce binding ~43-fold ($\Delta\Delta G$ = 2.2 kcal/mol). Taken together, these data clearly demonstrate that the highly conserved amino acids E31, L91, and K124 make substantial energetic contributions to the binding of SmpB to tmRNA and contribute directly to the formation of the ribonucleoprotein complex that recognizes stalled ribosomes.

Thermoanaerobacter tengcongensis (Tten) SmpB binds Tten tmRNA with nanomolar affinity

SmpB protein and tmRNA are conserved in Eubacteria. Therefore, it might be reasonably inferred that the specific contact residues implicated in the binding of *E. coli* SmpB protein to tmRNA might be similarly involved in the interactions of SmpB-tmRNA orthologues of other bacterial species. To evaluate the validity of this inference, I studied the interaction of *T. tengcongensis* (Tten) SmpB and tmRNA orthologues. Towards this end, I cloned, expressed and purified wild-type Tten SmpB protein and determined its affinity for Tten tmRNA^{112}. This Tten tmRNA RNA variant, like the tmRNA^{113} variant used for analysis of the *E. coli* SmpB –tmRNA interaction, contains the tRNA-like domain, a segment flanking the tRNA-like domain and is linked with a short tetraloop.

Electrophoretic mobility shift assay analysis showed Tten SmpB^{WT} to be capable of binding Tten tmRNA with high affinity, with K_d value of ~11 nM (Table 3.2). The binding assays were conducted in the presence of 100-fold excess competitor RNA and 300 mM KCl. The presence of high concentration of salt and non-specific competitor RNA indicates that the Tten SmpB binds Tten tmRNA with highly affinity and specificity (Figure 3.6).

Mutations in conserved Tten SmpB amino acids E28, L87, H89 and K120 cause a decrease in the affinity of Tten SmpB for tmRNA *in vitro*

When comparing the amino acid conservation profiles of SmpB protein, I found some intriguing differences between *E. coli* and Tten SmpB proteins. Namely, of the 115

sequences analyzed the *E. coli* SmpB residues N93 and Q94 are only marginally represented, 16% and 7% respectively. The corresponding Tten SmpB amino acid residues H89 and R90 are more strongly conserved, 66% and 33% respectively. This divergence could be of functional significance and hence permit investigation of subtle differences and intricacies of SmpB-tmRNA interaction across different bacterial species. Having established interaction parameters for Tten SmpB-tmRNA, I wished to determine if residues E28, L87, H89, R90 and K120, which correspond to residues E31, L91, N93, Q94 and K124 of *E. coli* SmpB, make similar contributions to binding of Tten SmpB protein to Tten tmRNA. To this end, I constructed single alanine substitution mutants of residues E28, L87, H89, R90 and K120 of Tten SmpB protein. Expression and purification of the Tten SmpB alanine mutants followed the same primary protocol as for wild-type Tten SmpB and the mutant proteins were purified to homogeneity as detected by Coomassie-stained SDS-PAGE gel electrophoresis (see Materials and Methods). The equilibrium dissociation constants (K_d) of each protein for interaction with the Tten tmRNA variant ($tmRNA^{112}$) were determined using electrophoretic mobility shift assays. All experiments were performed at least in triplicate and data were analyzed as described earlier. Figure 3.7 shows the binding data and the fitted curves for the representative Tten SmpB mutant proteins.

My analysis demonstrates that the single alanine substitution mutant $SmpB^{K120}$ makes the most significant contributions, as single alanine substitution at this position severely impaired binding of SmpB protein to tmRNA (Table 3.2 and Figure 3.7). The equilibrium dissociation constant dropped from ~14 nM for wild-type SmpB protein to ~581 nM for K120A, a greater than 40-fold loss of affinity for the $SmpB^{K120A}$ mutant.

This loss in binding affinity corresponds to a binding energy difference of 2.2 kcal mol^{-1} ($\Delta\Delta G$ = 2.2 kcal mol^{-1}) (Table 3.2). Amino acids L87 and H89 also contribute significantly to binding tmRNA RNA. Single alanine substitution at either residue decreased binding affinity for tmRNA RNA by ~20-fold (Figure 3.7, and Table 3.2). These losses in binding affinity correspond to a binding energy difference of 1.8 kcal mol^{-1} ($\Delta\Delta G$ = 1.8 kcal mol^{-1}). Alanine substitution at residue E28 decreased binding affinity by ~8-fold ($\Delta\Delta G$ = 1.2 kcal mol^{-1}). Analysis of Tten SmpB mutant with alanine substitution at residue R90 revealed that this amino acid also makes a modest contribution to Tten SmpB-tmRNA binding affinity ($\Delta\Delta G$ = 0.91 kcal mol^{-1}). These data clearly demonstrate that these highly conserved surface residues of Tten SmpB protein, akin to the corresponding *E. coli* SmpB residues, play a crucial role in binding of SmpB protein to tmRNA. Additionally, the pattern of energetic contribution from each individual amino acid in Tten SmpB is similar to the pattern of energetic contribution of the corresponding amino acids in *E. coli* SmpB. Contributions made by amino acids E28, L87, H89, R90 and K120 also correlate with the degree of conservation of these specific residues. Tten SmpB amino acids H89 and R90, which are respectively 66% and 33% conserved, make greater energetic contributions to tmRNA binding affinity of Tten SmpB than the corresponding *E.coli* SmpB residues N93 and Q94.

3.5 Discussion

I have identified critical amino acids of the SmpB protein that play a role in its function of binding tmRNA. Mutations in these specific residues drastically decrease the affinity of SmpB for Tten tmRNA[112] *in vitro*. I have defined the energetic contributions of these amino acids that are playing an important role in binding tmRNA. I present the first direct biochemical evidence that these amino acids are playing a significant role in tmRNA binding.

This electrophoretic mobility shift data (Table 3.1 and Figure 3.4) demonstrates that the amino acid residues E31, L91, N93 and K124 are paramount to the binding of *E. coli* SmpB protein to *E. coli* tmRNA. The decrease in affinity of these SmpB mutants for tmRNA also closely mirrors the defects that I see in endogenous tagging. The loss of multiple specific contacts leads to a decrease in the amount of tmRNA-tagged proteins (Figure 3.2C), and in the ability of SmpB to allow the stable association of tmRNA with 70S ribosomes (Figure 3.3B). It is clear that the critical first step in *trans*-translation, binding of SmpB to tmRNA is impaired in these SmpB variants. The decrease in affinity of *E. coli* SmpB for tmRNA is due to the loss of specific SmpB amino acids involved in binding tmRNA. This decrease in affinity for tmRNA leads to a decrease in the ability of SmpB to bring tmRNA to the ribosome and allow its stable association with 70S ribosomes. This decrease in tmRNA present at ribosomes results in a loss of endogenous tagging *in vivo*.

The highly conserved nature and spatial distribution of the amino acids E31, L91 and K124 on SmpB protein (Figure 3.1) suggested that these amino acids have been

conserved for a functional reason. It is now clear, from this direct biochemical data that these amino acids play an important role in SmpB binding to tmRNA. Mutation of these specific amino acids leads to a significant decrease in affinity of SmpB protein for tmRNA *in vitro*. The loss of binding affinity that I see *in vitro* clearly has an affecton *trans*-translation *in vivo*. Those mutants with the greatest decrease in binding affinity LK and EK show a decrease in endogenous tagging as well as a decreased amount of tmRNA associating with 70S ribosomes.

The equivalent amino acids (E26, L86, H88, K89 and K119 from *A. aeolicus*) have been highlighted by Guttman et al. based on their degree of conservation, but this is the first direct biochemical evidence demonstrating the significance of these SmpB amino acids in binding tmRNA. These are also the first direct measurements that clearly define the energetic contributions of each amino acid to the ribonucleoprotein complex formation between *E. coli* SmpB and *E. coli* tmRNA.

To further investigate the interactions involved in SmpB-tmRNA binding I wanted to characterize the binding of SmpB to tmRNA from a thermophilic bacterium. Studying the interactions between SmpB protein and tmRNA from a thermostable bacterium has a number of advantages. All of the structural data available on SmpB and tmRNA, both NMR data and crystallographic data is derived from thermophilic bacteria(40, 59, 133). Also, studying a more homologous SmpB protein to the thermophilic bacteria for which there is structural data may help to give us some insight as to the molecular nature of the interactions occurring between SmpB and tmRNA. I chose the bacterium *Thermoanerobacter tencongensis* (Tten), a thermostable bacterium with a greater number of homologous amino acid residues to *Aquifex aeolicus* as

compared to *E. coli* (Figure 3.1B). Tten shares 66.9% primary sequence homology with *Aquifex aeolicus* on the other hand *E.coli* only shares 58% homology with *Aquifex aeolicus.*

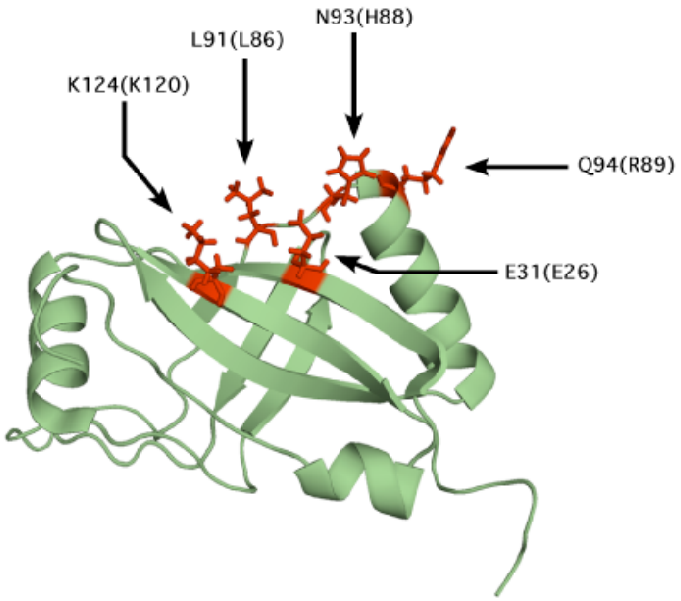
From this data, amino acids E31, L91 and K124 found on β2, β5, and β7 are significantly involved in binding tmRNA. The energetic contributions provided by E31, L91, and K124 are consistent with their degree of conservation. Amino acids L91 and K124 that are providing the most significant energetic contributions of 1.56 $kCalM^{-1}$ and 1.46 $kCalM^{-1}$ respectively are also the most highly conserved, 100% and 99% respectively. The conserved amino acid E31 contributes 1.14 $kCalM^{-1}$ to the binding energy and is 93% conserved.

To gain insight into the tmRNA nucleotides that might be involved in interactions with the E31, L91, N93, Q94, and K124 residues I analyzed the available crystal structures(40, 41). The tmRNA binding surface makes contacts within the tRNA like domain of tmRNA, specifically to nucleotides within the D-loop. Some of these interactions are likely between E31 interacting with C17, G18, L91 interacting with U16, A19, N93 interacting with C17, G18, Q94 interacting with A19 and K124 interacting with U16, G18. Biochemical analysis of the role of these nucleotides would yield insight into the role these nucleotides play in tmRNA binding.

3.6 Figures

Figure 3.1:

A.



B.

	31	91 93 94	124
E. coli	EEFEAGL...	TRKLLLNQRELD....	AWCKVKI...
T. tengcon.	ETYEAGI...	PRKLLLHRHEIN....	GLV KVEL...
A. aeolicus	ETYEAGI...	KRKLLLHKREIM...	NKV KVLI...
	*	* **	*

Figure 3.1: (A) Ribbon drawing of the tertiary structure of wild-type *A. aeolicus* SmpB. Amino acids highlighted in *red* were mutated to alanine and are depicted in a ball-and-stick representation. The ribbon diagram of the energy-minimized averaged NMR solution structure of Dong et al. (PDB 1K8H) was prepared using Pymol. (B) Selected aligned segments of primary amino acid sequences of SmpB protein from *E. coli*, Tten, and *A. aeolicus*. The *E. coli* numbering above the sequences and the asterisks (*) below the sequences highlight key amino acids mutated in this study.

Figure 3.2

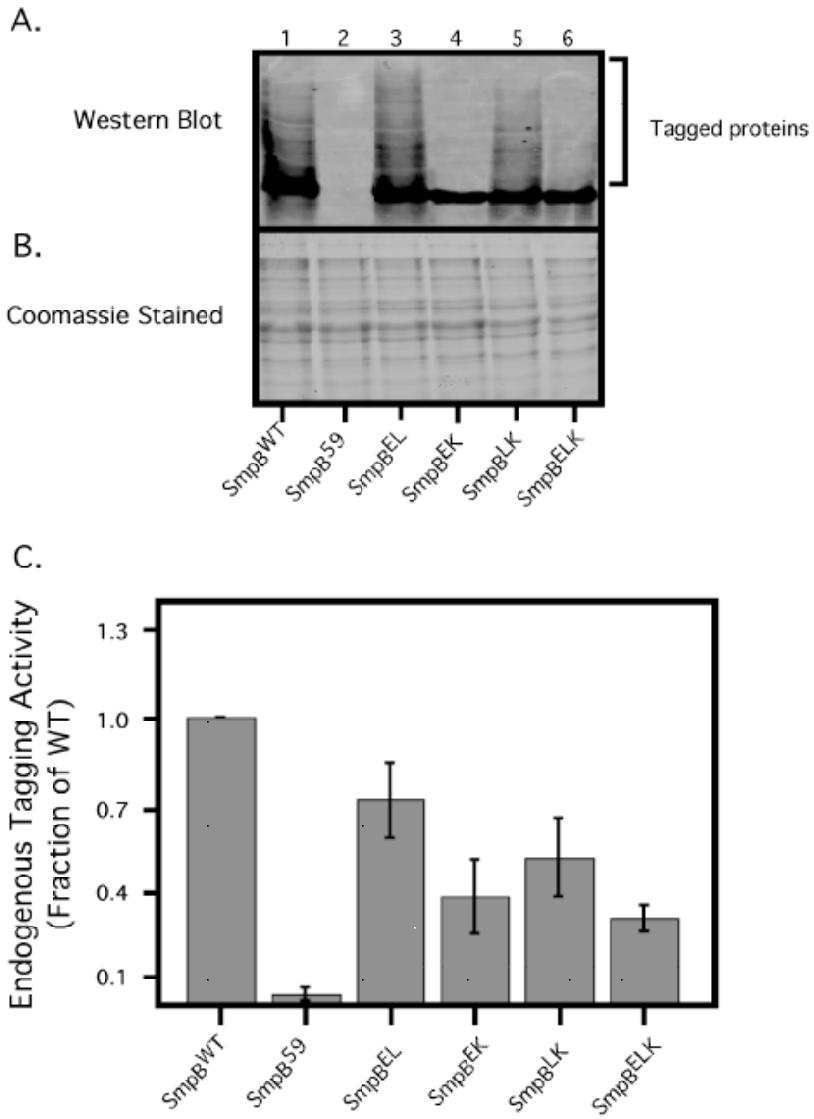


Figure 3.2: Analysis of the endogenous tagging activity of *E. coli* SmpB alanine-substitution mutants. $SmpB^{EL}$, $SmpB^{EK}$, $SmpB^{LK}$, $SmpB^{ELK}$ represent $SmpB^{E31A/L91A}$, $SmpB^{E31A/K124A}$, $SmpB^{L91A/K124A}$ and $SmpB^{E31A/L91A/K124A}$ respectively (A) A representative Western blot, developed with antibodies to the 6-His epitope, displaying all of the endogenously tagged proteins. (B) Coomassie stained gel showing that equal amounts of protein were loaded in each lane. (C) Bar graphs depicting the mean and standard deviation of 3 independent tagging assays displaying the level of tagging activity as compared to wild-type SmpB protein.

Figure 3.3

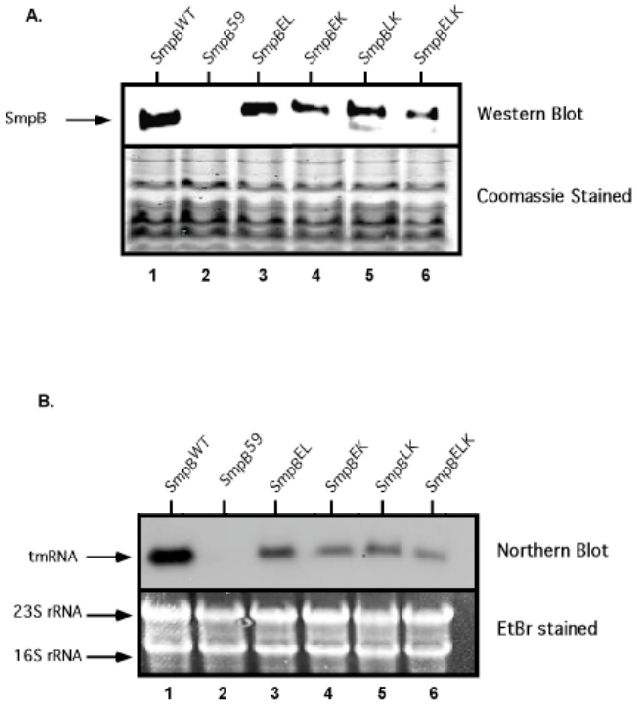


Figure 3.3: Ribosome-association assays. (A) Western-blot analysis with anti-SmpB antibodies displaying the amount of wild-type SmpB and select SmpB alanine substitution mutants associated with 70S ribosomes *in vivo*. Coomassie stained SDS-PAGE of sample in panel A to demonstrate equal amounts of total protein were loaded in each lane. (B) Northern blot analysis with a tmRNA-specific probe to detect 70S ribosome associated tmRNA. Ethidium bromide staining of the same gel as in *A* and *C*, to demonstrate that similar amounts of ribosomal RNA were loaded in each lane. The SmpB variant expressed in the cells from which the ribosomes were purified is indicated on top of each panel. $SmpB^{EL}$, $SmpB^{EK}$, $SmpB^{LK}$, $SmpB^{ELK}$ represent $SmpB^{E31A/L91A}$, $SmpB^{E31A/K124A}$, $SmpB^{L91A/K124A}$ and $SmpB^{E31A/L91A/K124A}$ respectively Wild-type SmpB is used as a positive control and $SmpB^{59}$, a nonfunctional truncated SmpB variant, is used as a negative control. Data and figure provided by Hye Jin Cho.

Figure 3.4

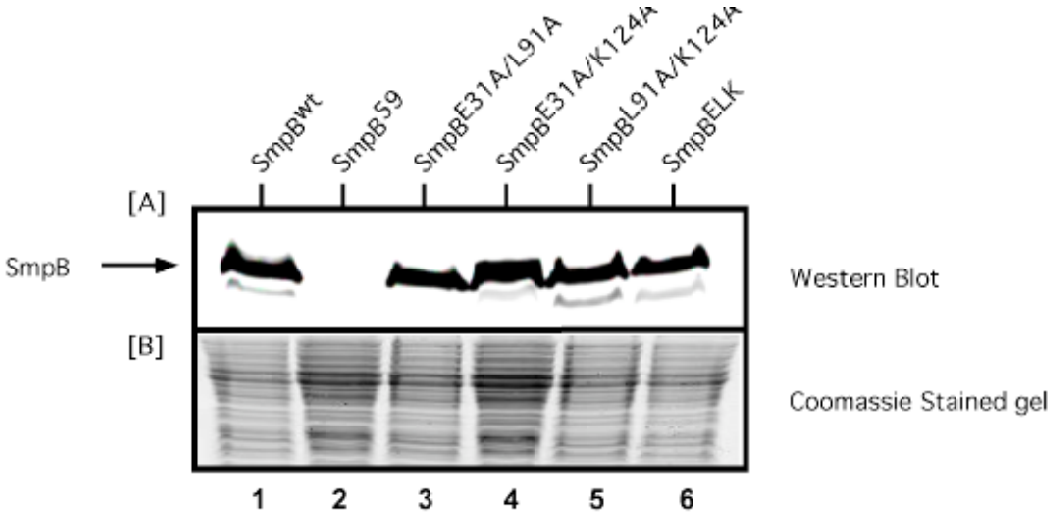


Figure 3.4: Analysis of the expression level of SmpB alanine-substitution mutants. (A) Western blot analysis with anti-SmpB antibodies showing the amount of each SmpB mutant present in the soluble S30 extract. (B) Coomassie stained SDS-PAGE of the 30S extract of the samples shown in panel A to demonstrate that equal amounts of protein were loaded per lane. The SmpB variant expressed in the cells from which the ribosomes were purified is indicated on top of each panel. $SmpB^{EL}$, $SmpB^{EK}$, $SmpB^{LK}$, $SmpB^{ELK}$ represent $SmpB^{E31A/L91A}$, $SmpB^{E31A/K124A}$, $SmpB^{L91A/K124A}$ and $SmpB^{E31A/L91A/K124A}$ respectively Wild-type SmpB is used as a positive control and $SmpB^{59}$, a nonfunctional truncated SmpB variant, is used as a negative control. Figure and data provided by Hye Jin Cho.

Figure 3.5

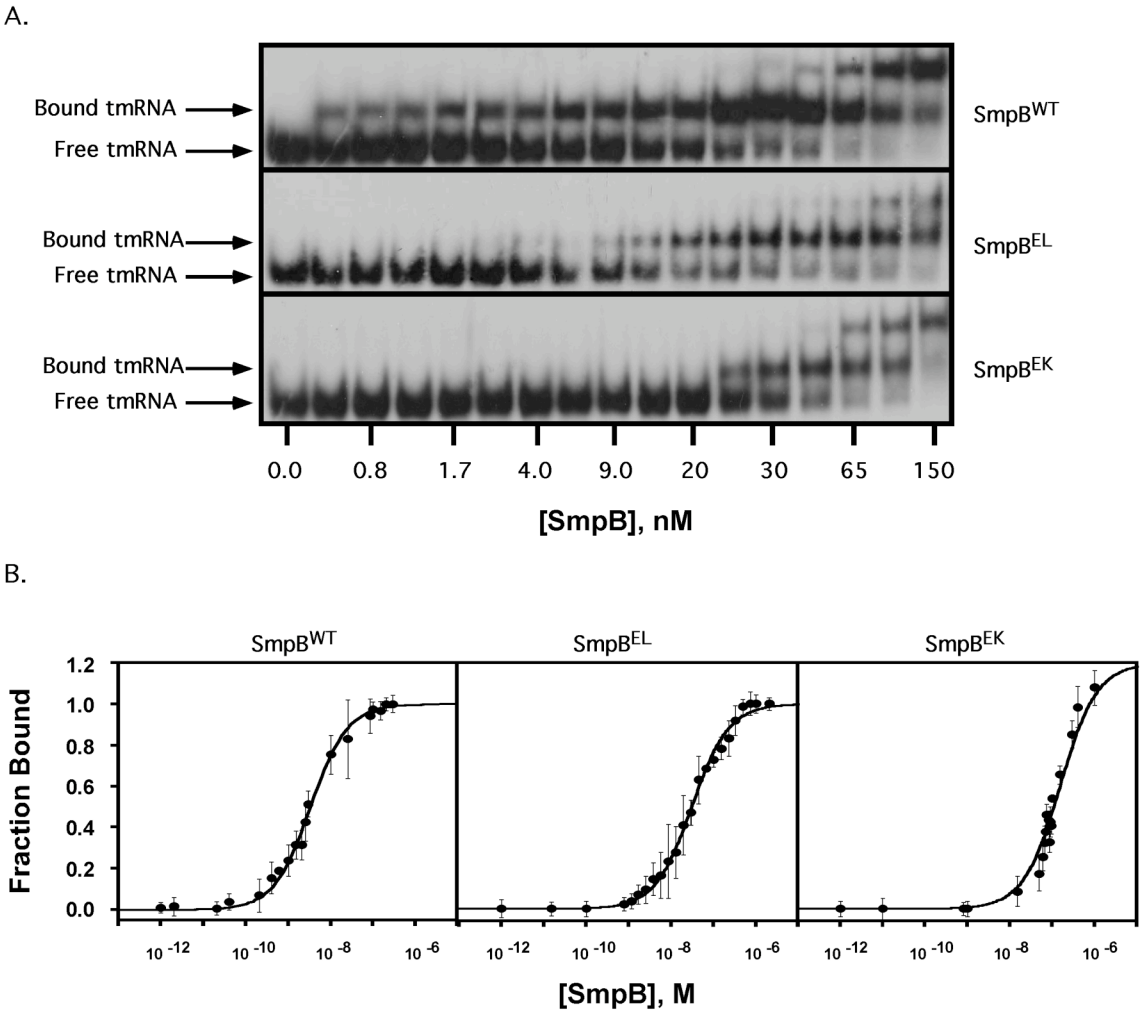


Figure 3.5: tmRNA-binding assays with *E. coli* SmpB alanine-substitution mutants. (A) Electrophoretic gel mobility-shift assays of the binding propensities of $SmpB^{wt}$, $SmpB^{E31A/L91A}$, $SmpB^{E31A/K124A}$ to *E. coli* $tmRNA^{113}$. The position of free and SmpB bound tmRNA on the autoradiogram is indicated by arrows to the left of each panel. (B) Curve-fit analysis of gel mobility-shift data of the SmpB mutants shown in panel A was used to determine the apparent equilibrium dissociation constants (see Table 3.1).

Figure 3.6

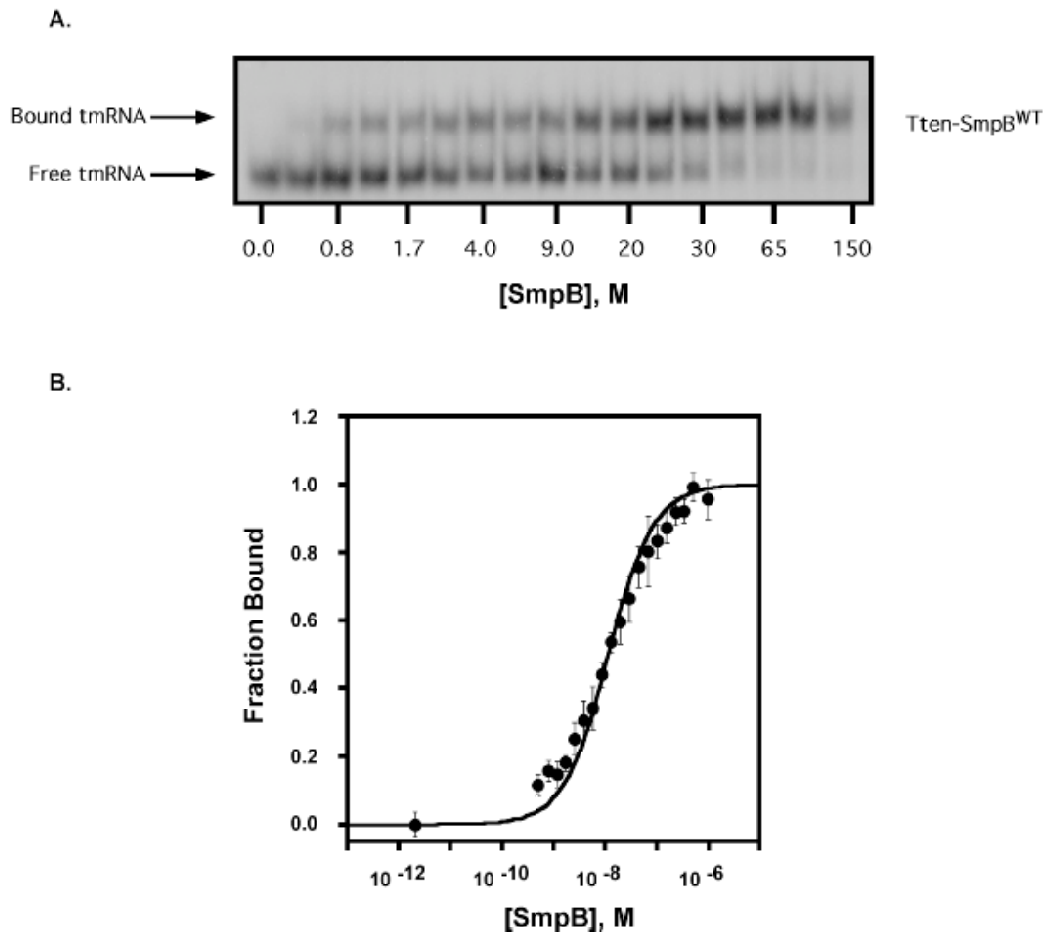


Figure 3.6: tmRNA-binding assay with wild-type *T. tengcongensis* (Tten) SmpB protein. (A) A representative autoradiogram of gel mobility-shift assay of the binding propensity of Tten-SmpB^{WT} to Tten-tmRNA^{112} RNA. (B) Curve-fit analysis of gel mobility-shift data of the Tten-SmpB^{WT} shown in panel A was used to determine its apparent equilibrium dissociation constant (see Table 3.2).

Figure 3.7

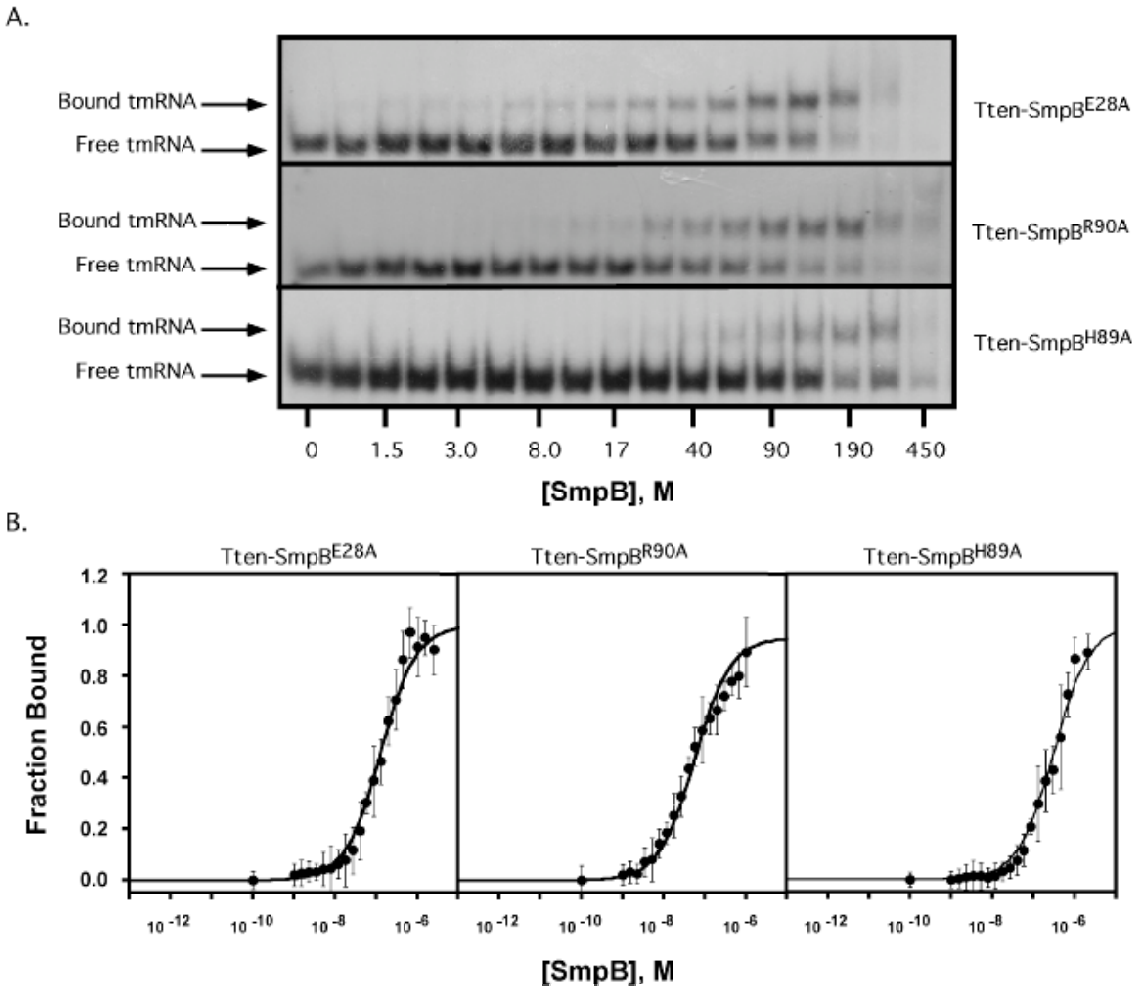


Figure 3.7: tmRNA-binding assay with *T. tengcongensis* (Tten) SmpB alanine-substitution mutants. (A) Electrophoretic gel mobility-shift assays of the binding propensities of $SmpB^{E28A}$, $SmpB^{R89A}$, and $SmpB^{H89A}$ to Tten-$tmRNA^{112}$. (B) Curve-fit analysis of gel mobility-shift data of the Tten-SmpB mutants shown in panel A used to determine the apparent equilibrium dissociation constants of these mutants (see Table 3.2).

3.7 Tables

Table 3.1.

tmRNA binding parameters, derived from gel mobility-shift assays, for wild-type *E. coli* SmpB and all alanine substitution variants.

E. coli *SmpB*	Kd (M)	ΔG (kcal mol^{-1})	ΔΔG (kcal mol^{-1})
Wt	$4 \times 10^{-9} \pm 1 \times 10^{-9}$	-11.4	
E31A	$29 \times 10^{-9} \pm 3 \times 10^{-9}$	-10.3	1.2
L91A	$58 \times 10^{-9} \pm 5 \times 10^{-9}$	-9.9	1.6
N93A	$26 \times 10^{-9} \pm 2 \times 10^{-9}$	-10.3	1.0
Q94A	$7 \times 10^{-9} \pm 2 \times 10^{-9}$	-11.1	0.3
K124A	$49 \times 10^{-9} \pm 3 \times 10^{-9}$	-10.0	1.5
E31A/L91A	$31 \times 10^{-9} \pm 4 \times 10^{-9}$	-10.2	1.2
L91A/K124A	$87 \times 10^{-9} \pm 5 \times 10^{-9}$	-9.6	1.8
E31A/K124A	$140 \times 10^{-9} \pm 5 \times 10^{-9}$	-9.4	2.1
E31A/L91A/K124A	$174 \times 10^{-9} \pm 23 \times 10^{-9}$	-9.2	2.2

Table 3.2.

tmRNA binding parameters, derived from gel mobility-shift assays, for wild-type *T. tengcongensis* SmpB and all alanine substitution variants.

T. tengcongenesis SmpB	K_d (M)	ΔG (kcal mol^{-1})	$\Delta\Delta G$ (kcal mol^{-1})
Wt	$12 \times 10^{-9} \pm 2 \times 10^{-9}$	-10.8	
E28A	$120 \times 10^{-9} \pm 10 \times 10^{-9}$	-9.4	1.3
L87A	$350 \times 10^{-9} \pm 40 \times 10^{-9}$	-8.9	1.8
H89A	$290 \times 10^{-9} \pm 18 \times 10^{-9}$	-8.9	1.8
R90A	$50 \times 10^{-9} \pm 6 \times 10^{-9}$	-9.8	0.9
K120A	$550 \times 10^{-9} \pm 50 \times 10^{-9}$	-8.5	2.2

Chapter 4: Identification of Critical SmpB Amino Acids in the Proximal C-terminal Tail

4.1 Summary

The SmpB protein is conserved throughout bacteria and is required for all known activities of tmRNA. A random mutagenesis approach was undertaken to identify SmpB amino acids that are critical for the function of SmpB. Using this approach Gly-132 was found to be a critical SmpB amino acid. Interestingly, this amino acid can be replaced by small uncharged amino acids and the function of SmpB can be recovered. I also found that Gly-132 is surrounded by a number of highly conserved charged amino acids, Lys-131, Lys-133 and Lys-134. These amino acids were also found to be critical for the function of SmpB. These amino acids are not involved in SmpB-tmRNA binding and are also not critical for SmpB-tmRNA-ribosome interactions contrary to previous suggestions (61). These SmpB amino acids are located at the base of the SmpB C-terminal tail and I propose that this region is likely involved in interactions that position or anchor the C-terminal tail within the ribosome.

4.2 Introduction

Small protein B (SmpB) is essential for all of the known functions of tmRNA. SmpB binds to tmRNA with high affinity and high specificity and is required for the stable association of tmRNA with ribosomes(53, 54, 57, 93). SmpB also plays a critical

role in *trans*-translation that is post ribosome binding but prior to the addition of the tmRNA-alanine charge(57). SmpB is found in all bacterial species, and its primary amino acid sequence is highly conserved(83). To identify SmpB amino acids critical for its function, SmpB was subjected to random mutagenesis and the mutants were screened for their ability to support *trans*-translation. I identified $SmpB^{G132D}$ as an SmpB variant unable to support *trans*-translation *in vivo*. I found that small uncharged amino acids could functionally substitute for glycine at this position. I considered that the highly conserved charged amino acids surrounding Gly-132, consisting of Lys-131, Lys-133 and Lys-134, might also be playing an important role in SmpB protein. Site-directed mutagenesis of these amino acids reveals that these amino acids are indeed of functional importance to SmpB protein. Mutation of these residues significantly decreases the level of endogenously tagged proteins *in vivo*. I demonstrate by electrophoretic mobility shift assay that mutation of these highly conserved amino acids does not affect SmpB-tmRNA binding. I also demonstrate that mutations that significantly decrease the level of endogenously tagged proteins do not affect the ability of SmpB to associate with 70S ribosomes contrary to previous suggestions (55, 61). I propose that this region of SmpB protein, at the base of the C-terminal tail, might play an important role in positioning this critical extension of the protein within the ribosomal A-site.

4.3 Experimental Procedures

$SmpB^{G132}$ Random Mutagenesis and screening

To generate a pool of random variants at amino acid position 132 of *E. coli* SmpB, plasmid pET28BA^{6xHis} was mutagenized using the Stratagene Quick Change Kit with the following primers

G132 Random Reverse 5'GTTTATCGTGCTGTTTCTTNNNTTTGGCGACGCCG,

G132Random Forward 5'CGGCGTCGCCAAANNNAAGAAACAGCACGATAAAC.

Mutagenesis reactions were treated with Dpn1(NEB) to digest template DNA. I generated a pool of 256 SmpB^{G132X} variants in E.coli strain W3110 Δ*smpB* Δ*ssrA*, these were patch plated onto fresh LB agar plates containing kanamycin. Clones where then screened by cross streaking using λ-*imm*p22 *c2-5dis* hybrid phage as previously described(47). A 2μl spot of phage at a 1x10^{-4} dilution was spotted onto LB agar plates and allowed to dry. The various SmpB clones were then streaked through the phage spot and incubated overnight at 37°C. The SmpB gene of pET28 BA^{6xHis} for all clones able to support phage lysis was sequenced and the nucleotide at position 132 identified. Several clones that were able to support the lytic phase of λ-*imm*p22 *c2-5dis* hybrid phage were also sequenced and characterized.

tmRNA^{6xHis} Tagging Assay

The endogenous tagging assay protocol used is a modification of Karzai et al. (6). 50mL cultures of either ΔssrA::cat, Δ*smpB*1(DE3) or Δ*smpB*Δ*ssrA* (DE3) with pETBA^{6xHis} were grown to an O.D.$_{600}$ of 0.800-.900 (when Δ*smpB*(DE3) or Δ*smpB*Δ*ssrA* (DE3) cells were used, cultures were held under constant induction using 1μM IPTG). Cells were harvested and resuspended in 1mL of lysis buffer (8M urea, 100mM potassium phosphate (pH8), 10mM Tris (pH8), and 5mM β-mercaptoethanol), and lysed

by rocking at room temperature for 1 hr. Cell debris was collected by centrifugation and the supernatant was added to 100μL of Ni-NTA agarose resin (Qiagen). Resin binding was allowed to proceed for 1 hour at RT. Resin-sample slurries were then applied to a mini-chromatography column (Biorad) and washed 4 times with 1mL of lysis buffer. Proteins were eluted in 150μL of elution buffer (8M urea, 100mM acetic acid, 20mM β-mercaptoethanol), and samples were resolved by electrophoresis on a 15% Tris-Tricine gel and Western blots were probed with a 1° Mouse α−6xHis antibody (Covance) and a 2° α−Mouse IR800 conjugated antibody (Molecular Probes). The 6xHis tagged proteins were detected using the IR dye conjugated antibody and the Odyssey Infrared Imaging System, and were quantified using Odyssey data analysis software (LI-COR).

Ribosome Association Assay

To prepare tight coupled 70S ribosomes, 750 ml of W3110 Δ*smpB*/(DE3) containing plasmid pET28 BA^{6xHis} or pET28 BA^{6xHis} with specified SmpB amino acid substitutions were grown in LB containing 1 μM IPTG, and 50μg/mL kanamycin to $O.D._{600}$ 0.800-1.00. Bacterial cells were harvested by centrifugation and stored at -80°C. Cell pellets were resuspended in Buffer A (20 mM Tris pH7.5, 300 mM NH_4Cl, 10 mM $MgCl_2$, 0.5 mM EDTA, 6 mM β-mercaptoethanol, 10 U/ml SuperASE In (Ambion) and lysed by sonication. Lysates were centrifuged at 33,000xg for 30 minutes. Supernatants were transferred to new tubes and centrifuged again at 33,000xg for 30 minutes. Typically, a 19 ml aliquot of the supernatant was layered onto a 32% sucrose cushion in buffer B (20 mM Tris (ph7.5), 500 mM NH_4Cl, 10 mM $MgCl_2$, 0.5 mM EDTA, 6 mM β-mercaptoethanol, 10U/ml SuperASE In (Ambion)) and centrifuged at 85,000 xg for 22

hrs. The pellet containing tight-coupled ribosomes was washed twice with 5ml of cold buffer B. Tight coupled 70S ribosomes were resuspended in 500ul (20 mM Tris (pH7.5), 100 mM NH_4Cl, 10 mM $MgCl_2$, 0.5 mM EDTA, 6 mM β-mercaptoethanol, 10U/ml SuperASE In (Ambion)) then analyzed by Western and Northern blotting. RNA for Northern blot analysis was extracted with Tri-LS reagent (Molecular Research) and equal amounts of RNA were loaded onto 1% formaldehyde agarose gels, transferred to Hi-Blot Nylon membrane (Amersham) and probed with a Psoralen-biotin (Ambion) labeled full length tmRNA specific DNA probe. For Western blot analysis equal numbers of ribosomes were loaded per lane and resolved by electrophoresis on 15% Tris-Tricine gels. Western blots were probed with rabbit polyclonal antibodies raised against purified SmpB protein and a secondary IR 800nm dye conjugated secondary anti-rabbit antibody (Molecular Probes).

Purification of SmpB protein Variants

Bacterial strain BL21 (DE3)pLysS (Stratagene) was transformed with plasmid pET28 BA^{6xHis} harboring $SmpB^{WT}$ or one of the alanine mutants: $SmpB^{N17A/K18A/R19A}$, $SmpB^{K131A/K133A/K134A}$ and $SmpB^{G132D}$. Typically, cells were grown in three liters of LB at 37° to an $O.D._{600}$ of 0.450~0.500 and induced with 1 mM IPTG for 3 hours. Cells were harvested and resuspended in lysis buffer (1 M NH_4Cl, 150 mM KCl, 50 mM Tris (pH 8.0), 2 mM β-mercaptoethanol, 20 mM immidazole) and lysed by sonication (3, 30 second pulses, with the addition of 0.1 ml of 0.1M PMSF after each pulse). Lysates were centrifuged for 1 hour at 13,000 rpm in an SS-34 rotor (Sorvall). Supernatants were mixed with 2 ml Ni^{2+}-NTA resin (Qiagen) pre-equilibrated in lysis buffer and binding

took place for 1 hour at 4°C with rocking. The Ni^{2+}-NTA resin was applied to a chromatography column and washed 3X with 30 ml lysis buffer. Proteins were eluted three times with 2 ml of elution buffer (150 mM KCl, 50 mM Tris (pH8.0), 200 mM Immidazole, 20 mM β-mercaptoethanol). The eluate was then diluted in FPLC buffer A (50 mM KCl, 50 mM HEPES (pH 7.5), 5 mM $MgCl_2$, 2 mM β-mercaptoethanol) and loaded onto a MonoS ion-exchange column (Amersham-GE-Healthcare). A gradient of 50 mM KCl to 850 mM KCl was developed over 20 column volumes to isolate the SmpB protein. SmpB protein, with greater 95% purity, elutes at approximately 500 mM KCl under these conditions. Protein concentrations were determined by absorbance at 280 nM, using an SmpB extinction coefficient of 29575 M^{-1} cm^{-1} for *E. coli* $SmpB^{WT}$. Protein aliquots were stored at -80°C.

Electrophoretic Mobility Shift Assays

tmRNA variants used in this study were labeled at the 3'-end as previously described(54, 57). Electrophoretic mobility shift assays (EMSA) were performed as described with minor modifications(54, 57). Briefly, *E. coli* SmpB protein was diluted to the desired concentrations in EMSA buffer (50 mM Tris (pH7.5), 2 mM $MgCl_2$, 300 mM KCl, 100 μg/ml bovine serum albumin, 2 mM β-mercaptoethanol 0.01% NP-40 (v/v), 10% glycerol (v/v)) in the presence of 100 nM total *E. coli* tRNA as a nonspecific competitor. Approximately 100 fmol of 3' end labeled $tmRNA^{113}$ RNA (~1000 cpm per reaction) was added to each tube and incubated at room temperature for 30 minutes. Samples were loaded on a 12% non-denaturing gel and resolved by electrophoresis at

200V in 0.5X TBE to separate the free RNA from RNA-protein complexes. Gels were run at 4°C, dried and exposed overnight to phosphoimager screens.

4.4 Results

SmpB Glycine 132 is a critical residue for tmRNA-SmpB mediated *trans*-translation *in vivo*

Research in the field of *trans*-translation has shown that SmpB protein is a requisite factor in the *trans*-translation ribosome rescue system(53, 54, 64). SmpB functions in a number of different phases in the *trans*-translation mechanism. SmpB binds specifically and with high affinity to the tRNA-like domain of tmRNA and is necessary for the recruitment and stable association of the tmRNA-SmpB complex with stalled ribosomes. It is also required for a novel uncharacterized function following ribosome association but prior to the addition of the tmRNA alanine charge (53, 54, 57, 64). Our lab is interested in investigating the roles played by SmpB protein in *trans*-translation. We have shown that SmpB is required for functional tmRNA ribosome association, identified residues within the C-terminal tail required for a novel function of SmpB protein and established that highly conserved surface exposed amino acids are critical for SmpB binding to the tRNA-like domain of tmRNA (53, 54, 57, 64). I set out to further elucidate the specific roles of SmpB in *trans*-translation by identifying SmpB amino acids that are critical for *trans*-translation. To this end, I performed a random mutagenesis screen of SmpB protein. Plasmid pET28BA, a plasmid harboring the wild-

type *E. coli smpB* and *ssrA* genes was subjected to hydroxylamine mutagenesis and the mutagenized vector was transformed into W3110Δ*smpB* Δ*ssrA*. To identify clones unable to support *trans*-translation the transformants were patch plated and subsequently screened by λ*imm*p22 *c2-5dis* hybrid phage cross streak assay(47).

SmpB and tmRNA are required for the lytic induction of the λ*imm*p22 *c2-5dis* hybrid phage (Figure 4.1A)(94, 138). I utilized this phenotype to identify pET28BA variants generated by chemical mutagenesis that are unable to support *trans*-translation *in vivo*. Clones unable to support *trans*-translation *in vivo* were identified by their resistance to lysis when streaked across λ*imm*p22 *c2-5dis* hybrid phage. Using this methodology, several pET28BA clones were identified that were unable to support the lytic cycle of λ*imm*p22 *c2-5dis* hybrid phage (data not shown). The *smpB* and *ssrA* genes of the pET28BA plasmid contained in these clones were sequenced and the nature of the mutation was identified. With the exception of one SmpB variant, $SmpB^{G132D}$, all of the clones identified contained premature stop codons within the *smpB* gene. Premature stop codons within the *smpB* reading frame resulting in a truncation of SmpB protein within the first 153 amino acids renders the protein nonfunctional(64). The SmpB variants with premature stop codons I identified were not pursued. I did however identify an SmpB variant, $SmpB^{G132D}$, resulting from a missense mutation, replacing a glycine residue at position 132 with aspartic acid. This SmpB variant was unable to support the lytic cycle of λ*imm*p22 *c2-5dis* hybrid phage, (Figure 4.1B) suggesting that the $SmpB^{G132D}$ variant was unable to support a critical function of the SmpB protein in *trans*-translation.

A possible explanation of this phenotype was that this mutation affects the expression of SmpB protein. To test this I analyzed the expression level of $SmpB^{G132D}$

from pET28BA SmpB^{G132D} by Western blotting. I found that SmpB^{G132D} was present at the same level as SmpB^{WT} from pET28BA. The SmpB^{G132D} variant also remained exclusively in the soluble fraction and did not aggregate. (Data Not Shown).

Glycine 132 is not required for SmpB function *in vivo*

Glycine 132 can be functionally substituted *in vivo* by small amino acids

Multiple sequence alignment of the SmpB protein amino acid sequence revealed that the glycine at position 132 is 98% conserved (Figure 4.2A). Within the 113 sequences analyzed, glycine at position 132 is only replaced in *Mycoplasma genitalium* and *Mycoplasma pneumoniae* by a proline residue. To gain further insight into the role of Gly-132 in *trans*-translation I identified amino acids that could functionally substitute for glycine at this position. To this end, I used random PCR mutagenesis to randomize the glycine 132 codon in plasmid pET28BA^{His6}. This plasmid harbors the *smpB* gene and the *ssrA* gene with an altered mRNA-like domain of tmRNA, replacing the degradation tag (ANDENYALAA) with a stabilizing six-histidine tag (ANDEHHHHHH). The tmRNA^{His6} variant has no affecton the λ*imm*p22 *c2-5dis* hybrid phage phenotype and using the λ*imm*p22 *c2-5dis* hybrid phage assay I screened for functional SmpB^{G132X} variants.

I screened 256 clones for their ability to lyse when streaked through λ*imm*p22 *c2-5dis* hybrid phage. My screen had a background of 4%, based on the number of clones obtained in my control reaction versus the 256 clones generated by my PCR mutagenesis (10/256 clones). Out of 256 clones, a total of 45 clones lysed when streaked through

λ*imm*p22 *c2-5dis* hybrid phage, 36 of which gave a very strong complete lysis phenotype and 9 clones gave a moderate lysis phenotype. These clones were referred to as the complementing clones. These complementing clones were sequenced and the amino acid at position 132 was identified. I identified six amino acids at position 132 that allowed for the lytic induction of λ*imm*p22 *c2-5dis* hybrid phage. Glycine, alanine, serine, histidine, phenylalanine, and arginine were all identified in the complimenting clones with the majority of the clones identified, 33 out of the 45, coding for glycine. The $SmpB^{WT}$ glycine codon (GGT) as well the GGA and GGG codons were represented multiple times. I did not however obtain any clones with the 4th glycine codon, GGC. To investigate if there was a codon bias at this position I introduced the GCG codon by site-directed mutagenesis and this SmpB variant, $SmpB^{G132G(GCG)}$, also allowed lytic induction of the λ*imm*p22 *c2-5dis* hybrid phage. I also introduced a proline residue at position 132 to analyze if the only other naturally occurring amino acid found at this position could functionally substitute for Gly-132 in *E. coli* and support lysis by λ*imm*p22 *c2-5dis* hybrid phage. Interestingly, this SmpB variant gave a moderate lysis phenotype indicating that it was not able to fully support the functions of SmpB protein. These SmpB variants $SmpB^{G132(GCG)}$ and $SmpB^{G132P}$ were grouped with the complementing clones though not originally pulled out in my screen.

I also analyzed a selection of the non-complementing clones that were unable to support the lytic induction of λ*imm*p22 *c2-5dis* hybrid phage. I identified leucine, valine, threonine and glutamine as amino acids unable to functionally substitute for Gly-132 in the λ*imm*p22 *c2-5dis* hybrid phage assay. These results suggest that there is not a strict requirement for glycine at position 132 though the glycine appears best fit for this

position with other substituting amino acids giving a moderate λ*imm*p22 *c2-5dis* hybrid phage phenotype.

SmpB Gly-132 variants can effectively support the tagging function of *trans*-translation

To further evaluate the complementing and non-complementing SmpB variants I analyzed the ability of these SmpB variants to support the tagging function of the SmpB-tmRNA *trans*-translation system. I utilized the pET28BA^{His6} plasmid containing an altered mRNA-like region of tmRNA that allows for the addition of a stabilizing six-histidine tag (ANDEHHHHHH), in place of the degradation tag (ANDENYALAA). This allows tagged substrates of the *trans*-translation system to be purified by Ni-NTA chromatography, resolved by SDS-polyacrylamide electrophoresis and analyzed by Western blotting using anti-His-6 antibodies. The saturation PCR mutagenesis of glycine at position 132 of *smpB* was performed on pET28BA^{His6} enabling us to easily analyze the SmpB variants identified in the phage screen for their ability to tag endogenous proteins in W3110 *ΔsmpBΔssrA*. All of the complementing and non-complementing clones were analyzed for their ability to support the endogenous tagging function of the *trans*-translation system. Their ability to tag endogenous proteins was compared to SmpB^{WT} and SmpB^{59} a truncated SmpB variant that is nonfunctional and unable to support the tagging activity of the *trans*-translation system(54, 64).

My analysis of the ability of the complementing clones to support the *trans*-translation tagging activity revealed that there is no codon bias for the SmpB^{WT} (GGT)

codon. Regardless of the glycine codon used, tagging was restored to 100% of SmpB^{WT} levels (Figure 4.3A, B and data not shown). Also, the SmpB^{G132A} and SmpB^{G132S} variants were able to effectively replace glycine at position 132 of SmpB protein. These SmpB variants tag endogenous proteins at or above 90% as compared to SmpB^{WT} protein. It is interesting however that out of the SmpB variants analyzed, only the SmpB$^{G132G(GGC)}$ variant was able to completely restore tagging activity to 100% as compared to SmpB^{WT} (Figure 4.3B Compare Smp^{WT} and SmpB$^{G132G(GCG)}$). The SmpB^{G132H} and SmpB^{G132P} variants tag endogenous proteins at similar levels, 81% and 77% respectively as compared to SmpB^{WT}. It was interesting to find replacing glycine with proline, the only other naturally occurring amino acid, was unable to fully complement the endogenous tagging activity. This tagging phenotype does mirror the inability of the SmpB^{G132P} variant to provide a strong λ*imm*p22 *c2-5dis* phage lysis phenotype. The final two clones analyzed in the set of complementing clones, SmpB^{G132F} and SmpB^{G132R} were able to support the tagging activity of *trans*-translation but at substantially reduced levels as compared to SmpB^{WT}. The tagging activity of the SmpB^{G132F} variant was reduced by 40% and the tagging activity of SmpB^{G132R} was reduced by 70% as compared to SmpB^{WT} levels.

Taken together, these results reveal that SmpB$^{G132G(GGC)}$ is fully capable of replacing SmpB^{WT} and there is no bias for the wild-type GGT codon at position 132 of *smpB*. There is however a preference for glycine at position 132, as only glycine was able to fully restore wild-type tagging activity. The SmpB^{G132A} and SmpB^{G132S} variants were able to restore the tagging activity close to wild-type levels but the histidine, proline, phenylalanine and arginine variants showed a dramatic decrease in the ability to

support the tagging activity of *trans*-translation. These results demonstrate that Gly-132 of SmpB is an important SmpB residue and that glycine at position 132 is optimally suited for this role for *E. coli* SmpB. Substitution of residue Gly-132 with other amino acids allow for tagging of endogenous proteins but at reduced levels.

I also analyzed the non-complementing clones $SmpB^{G132L}$, $SmpB^{G132V}$, $SmpB^{G132T}$ and $SmpB^{G132E}$ for their ability to support the *trans*-translation tagging activity (Figure 4.4A, B). The SmpB variants $SmpB^{G132V}$ and $SmpB^{G132T}$ are able to partially support the tagging of endogenous proteins but at greatly reduced levels as compared to $SmpB^{WT}$, both variants tag at levels reduced to 54% of wild-type levels. The SmpB variants $SmpB^{G132L}$ and $SmpB^{G132E}$ show severely reduced levels of endogenously tagged proteins. The $SmpB^{G132L}$ and $SmpB^{G132E}$ variants tag at 6% and 2% of $SmpB^{WT}$ levels, showing an almost complete loss of the ability of the *trans*-translation system to tag endogenous proteins. This data indicates that there appears to be a bias for small residues at this position (see discussion and Figure 4.4B) and glycine is best suited for this position on SmpB.

Conserved amino acids surrounding Gly-132 are critical for SmpB function in *trans*-translation

The chemical mutagenesis of SmpB and subsequent screening by the λ-*imm*p22 *c2-5dis* cross streak assay identified SmpB residue Gly-132 as playing a critically important role in *trans*-translation. Multiple sequence alignment revealed that Gly-132 is 98% conserved in the sequences analyzed and also revealed that residue Gly-132 is

surrounded by highly conserved charged residues Lys-131, Lys-133 and Lys-134 (Fig. 4.2A). The Lys-133 residue is the most highly conserved residue surrounding Gly-132, being conserved as lysine in 96% of the sequences analyzed and lysine or arginine in 98% of these sequences. Lysine 131 is conserved as lysine in 67% of the sequences analyzed and lysine or arginine in 96% of the sequences analyzed. The third highly conserved charged residue, Lys-134 is conserved as lysine in 70% of the sequences analyzed and as lysine or arginine in 82% of the sequences analyzed. The high degree of sequence conservation of these charged amino acids suggests that they are conserved for a functional purpose. In fact, it has been suggested that these residues play a role in RNA binding and most recently it has been speculated that these residues play an important role in SmpB binding to stalled ribosomes(55, 61). I also recognized a highly conserved region of SmpB on the amino terminus that is correspondingly as highly conserved as the Lys-131 to Lys-134 region. This region of SmpB consisting of residues Asn-17, Lys-18 and Arg-19 contains highly conserved charged residues and has also been suggested to play a role in RNA binding(55). I also wanted to analyze this region and compare it to the Lys-131 to Lys-134 region. The Asn-17 to Arg-19 region is very highly conserved, the asparagine at position 17 is 98% conserved, lysine or arginine at position 18 are 98% conserved and position 19 contains lysine or arginine in 89% of the sequences analyzed.

In determining the importance of Gly-132 I hypothesized that the entire Lys-131 to Lys-134 region might be important for the function of SmpB protein. The Asn-17 to Arg-19 region, being similarly conserved and suggested as a possible RNA binding surface, might also be playing a role in SmpB-tmRNA mediated *trans*-translation. To analyze the roles of these regions of SmpB protein I generated single, double and triple

alanine substitution variants of SmpB protein. I generated SmpB variants in both regions of SmpB protein Asn-17 to Arg-19 and Lys-131 to Lys-134. To investigate the role of these amino acids I again utilized the tmRNA^{His6} endogenous tagging assay.

The single, double and triple alanine substitution variants generated in pET28BA^{His6} were transformed into W3110 (DE3) *ΔsmpBΔssrA*. The endogenously tagged proteins were then purified by Ni-NTA chromatography, resolved by SDS-polyacrylamide electrophoresis and detected by Western blotting. The single alanine substitution variants of any of the residues analyzed did not show an appreciable decrease in the ability to tag endogenous proteins (Data not shown). Surprisingly, the triply substituted variant SmpB$^{N17A/K18A/R19A}$ did not show any decrease in its ability to support the tagging function of *trans*-translation (Figure 4.5A, B lane 3). The SmpB double and triple substitution variants of the Lys-131, Lys-133, and Lys-134 residues of SmpB did show a significant decrease in the ability to support the tagging function of *trans*-translation (Figure 4.5, Lanes 4-6). The double substituted variants SmpB$^{K131A/K133A}$ and SmpB$^{K131A/K134A}$ showed a 12% and 13% reduction in the ability to tag endogenous proteins. The most defective double-alanine-substitution variant was SmpB$^{K133A/K134A}$, which showed a greater than 30% reduction in the ability to tag endogenous proteins. The triple-alanine-substitution variant SmpB$^{K131A/K133A/K134A}$ had the most deleterious affect on endogenous tagging, a 75% reduction in the ability to tag endogenous proteins. These results demonstrate that the SmpB N17/K18/R19 region does not play a significant role in the ability of the SmpB-tmRNA mediated *trans*-translation system to tag endogenous proteins. These data also reveal that the region of SmpB protein containing the residues Lys-131, Lys-133, and Lys-134 play a significant role in the ability of the *trans*-

translation system to tag endogenous proteins. Taken together with the data from the $SmpB^{G132}$ region it is now clear that this region is very important in SmpB-tmRNA mediated *trans*-translation.

Substitution of the conserved amino acids N17, K18, R19 and K131, K133, K134 does not affect SmpB-tmRNA ribosome association

I have found that mutations in the conserved SmpB amino acids Lys-131, Gly-132, Lys-133 and Lys-134 have a deleterious affecton the function of SmpB and play an important role in facilitating SmpB-tmRNA tagging of endogenous proteins *in vivo*. These amino acids had previously been suggested to comprise an RNA binding surface, and more recently it has been speculated that these residues play a role in association of SmpB with ribosomes(61). If these conserved amino acids do play a role in tmRNA-SmpB-ribosome interactions, substitution of the charged residues with alanine would remove the interactions between the lysine side chains and rRNA or ribosomal proteins. Loss of SmpB-tmRNA ribosome association would lead to a decrease in the ability to tag endogenous proteins due to the inability of SmpB and tmRNA to stably associate with ribosomes. To test the hypothesis that these amino acids are playing a role in SmpB-tmRNA association with ribosomes, I purified 70S ribosomes from W3110 Δ*smpB*(DE3) harboring the SmpB variant $SmpB^{K131A/K133A/K134A}$ and assayed for the presence of SmpB and tmRNA with 70S ribosomes.

I also analyzed the SmpB variant $SmpB^{N17A/K18A/R19A}$ for its ability to stably associate with 70S ribosomes and deliver tmRNA to stalled ribosomes. The extremely

high degree of amino acid conservation within this area of SmpB protein suggests that these residues may be conserved for a functional purpose. Although the $SmpB^{N17A/K18A/R19A}$ triple-alanine-substitution variant had no affect on the ability of the *trans*-translation system to tag endogenous proteins, I considered the possibility that the tmRNA endogenous tagging assay may not detect a ribosome association deficiency and I analyzed the $SmpB^{N17A/K18A/R19A}$ variant along with $SmpB^{K131A/K133A/K134A}$ for the ability to associate with stalled ribosomes.

The 70S ribosomes were purified under high stringency conditions (See Materials and Methods) and the 70S ribosomes were probed by Western blotting for the presence of SmpB protein using polyclonal anti-SmpB antibodies. The results revealed that equal levels of SmpB protein were associated with 70S ribosomes in all of the SmpB variants tested (Figure 4.6A). As SmpB and tmRNA are both required at the ribosome for *trans*-translation to occur, I then assayed for the presence of tmRNA on the ribosome by Northern blotting. Northern blot analysis showed that tmRNA is also fully capable of associating with purified 70S ribosomes (Figure 4.6B). Taken together, these results indicate that the SmpB variants studied, $SmpB^{N17A/K18A/R19A}$, and $SmpB^{K131A/K133A/K134A}$ are fully proficient in their ability to associate with stalled ribosomes. Furthermore these SmpB variants are also fully capable of facilitating the association of tmRNA with ribosomes.

Previous literature has strongly speculated that the K131/K133/K134 region of SmpB protein is involved in SmpB-ribosome association(61). The SmpB variant $SmpB^{K131/K133/K134}$ might have a subtle ribosome association defect that was not detected by my analysis of 70S ribosomes. To explore this possibility I purified tight coupled 70S

ribosomes and incubated them in a buffer containing low magnesium (2mM $MgCl_2$) to promote ribosomal subunit dissociation. The 30S and 50S ribosomal subunits were then purified by gradient ultracentrifugation and SmpB was detected by Western blotting. I did not detect a difference between $SmpB^{WT}$, $SmpB^{K131A/K133A/K134A}$, and SmpB $^{N17A/K18A/R19A}$, all of the SmpB-tmRNA complexes were released from ribosomes upon dissociation into subunits (Data not shown). Taken together, this data suggests that the decrease in the level of endogenously tagged proteins is not due to an inability of these SmpB variants to associate with ribosomes. This data suggests that these conserved amino acids are not playing a vital role in SmpB-ribosome interactions facilitating SmpB-ribosome binding.

The SmpB residues Lys-131, Lys-133 and Lys-134 do not play a role in SmpB-tmRNA binding

It is clear that amino acid substitutions of the highly conserved SmpB Gly-132 residue do not support lytic induction of the λ-*imm*p22 *c2-5dis* hybrid phage (Figure 4.1B) and decrease the endogenous tagging function of *trans*-translation (Figure 4.3). Also, substitutions in the Lys-131 to Lys-134 region of SmpB have a deleterious affecton the ability of SmpB to support the tagging function of *trans*-translation (Figure 4.5). To further dissect the nature of this defect I wanted to determine if these defects were due to an inability of these SmpB variants to bind to the tRNA-like domain of tmRNA. Previously it has been shown that mutation of residues involved in binding the tRNA-like domain of tmRNA lead to a decrease in the ability of those SmpB variants to support the

tagging function of *trans*-translation, stemming from an inability to stably bind tmRNA(54).

To characterize the ability of the SmpB variants to bind tmRNA I analyzed SmpB-tmRNA binding by electrophoretic mobility shift assay (EMSA). I made use of a shortened version of tmRNA, $tmRNA^{113}$, consisting of only the tRNA-like domain of tmRNA. Based on biochemical data as well as the co-crystal structures of SmpB bound to tmRNA, the tRNA domain is sufficient and necessary for binding to SmpB(40, 41, 54).

The SmpB variants $SmpB^{K131A/K133A/K134A}$ and $SmpB^{G132D}$ were purified and the electrophoretic mobility shift assay was used to analyze their ability to bind $tmRNA^{113}$. Figure 4.7 shows that there is no difference in the binding pattern of $SmpB^{WT}$ compared to that of $SmpB^{K131A/K133A/K134A}$.

4.5 Discussion

SmpB plays an indispensable role in the *trans*-translation ribosome-rescue system. Our lab is interested in fully characterizing the dynamic roles played by SmpB protein during *trans*-translation. To this end, I have identified SmpB amino acids that are critical for the functions of SmpB to further elucidate the roles SmpB plays in *trans*-translation. I identified the highly conserved SmpB residue Gly-132 as well as the surrounding residues Lys-131, Lys-133 and Lys-134 as playing an important role in the tmRNA-SmpB mediated *trans*-translation system. My data suggests that these SmpB residues play an important role on SmpB protein, intriguingly these do not affect tmRNA-SmpB ribosome association or tmRNA-SmpB binding.

Random mutagenesis of SmpB protein followed by screening using λ*imm*p22 *c2-5dis* hybrid phage uncovered SmpB Gly-132 as a critical amino acid of SmpB protein (Fig. 1B). Knowing that the spatial positioning of this residue might give us some insight into its function, I analyzed the NMR structures of SmpB protein as well as the co-crystal structures of SmpB bound to the tRNA-like domain of tmRNA. My analysis revealed that glycine 132 is located in a region of SmpB protein beyond the final β-strand (β7 of *Aquifex aeolicus*), on the face of the protein opposite the surface responsible for binding the tRNA-like domain of tmRNA (Figure 4.2B)(40, 41, 55, 59). The region of SmpB protein containing Gly-132 is found in multiple positions within the various NMR structures, though always extending out from the core structure of SmpB protein, and is not in any secondary structural features in structures published to date. The $SmpB^{G132D}$ variant was soluble when expressed from pET28BA $SmpB^{G132D}$ and the purified protein bound the tRNA-like domain with an affinity similar to $SmpB^{WT}$. All of the SmpB variants in the Lys-131 to Lys-134 region of SmpB bound to the tRNA-like domain of tmRNA with similar affinities. Since the contacts necessary for SmpB-tmRNA binding are found throughout the primary amino acid sequence of SmpB protein I find it very unlikely that these protein variants are misfolding or have some global protein stability problem. Since these proteins are able to bind to the tRNA-like domain, it would seem reasonable to assume that they are retaining the necessary structure and contacts necessary for those interactions to occur. These results suggest that the Gly-132 residue does not play an important role in protein stability that would lead to misfolding and also that these residues do not play an important role in tmRNA-SmpB binding. Together,

these results suggest that Gly-132 plays an important role in *trans*-translation that is unrelated to the ability of SmpB to bind to the tRNA-like domain of tmRNA.

The exact cause of the λ*imm*p22 *c2-5dis* hybrid phage phenotype is currently a matter of debate in the field (139-141) but it has been speculated that the level of certain repressors are necessary to keep the phage in the lysogenic phase. The requirement for the SmpB-tmRNA system is suggested to play a role in the tagging and degradation of these factors. Although the $tmRNA^{His\text{-}6}$ variant adds a stabilizing six-histidine tag to the C-terminus of tagged proteins some of these tagged proteins might still be degraded. This low level of degradation might represent a threshold that is represented by the phage phenotype.

My analysis of the complementing and non-complementing SmpB variants, gave me insight into the role SmpB residue Gly-132 could be performing in *trans*-translation. It is clear from analysis of the ability of various $SmpB^{G132X}$ variants to support the tagging function of *trans*-translation that glycine is the only amino acid able to fully complement the tagging phenotype (Figure 4.3A, B). Other amino acids can replace Gly-132 and allow tagging of endogenous proteins but at reduced levels compared to $SmpB^{WT}$. When the complementing amino acids are arranged in order of decreasing mass, as in Figure 4.3, an interesting trend emerges. Amino acids that are small (Gly/Ala) or have shorter side chains are more acceptable at position 132 and allow for greater tagging of endogenous proteins (Figure 4.3 Compare Gly/Ala to Ser/His). Amino acids that are highly charged do not function well in this position. The $SmpB^{G132X}$ variants to His, Arg or Glu are deleterious to the tagging function of SmpB. Although I did not analyze the tagging phenotype of $SmpB^{G132D}$, due to the strong λ*imm*p22 *c2-5dis* hybrid phage

phenotype it is reasonable to assume that it has a tagging phenotype similar to SmpB^{G132E}. SmpB^{G132E} has a greater than 90% decrease in endogenous tagging. It is also interesting to compare the SmpB^{G132S} and SmpB^{G132T} variants. The SmpB^{G132S} variant tags endogenous proteins at close to 90% of wild-type levels while the SmpB^{G132T} variant tags endogenous proteins at 50% of wild-type levels. The addition of a methyl group (SmpB^{G132T} vs SmpB^{G132S}) leads to a 40% decrease in the ability to tag endogenous proteins. The picture that emerges from this data is that there appears to be a steric necessity for the amino acid at this position to be small and uncharged. Larger amino acids are not tolerated and neither are smaller charged amino acids (eg.SmpB^{G132D}). All of these SmpB variants are expressed equally well, fully soluble and remain exclusively in the soluble fraction (Data Not Shown). This data along with the *in vitro* binding data suggests that these SmpB variants do not appear to affect the overall structure of SmpB protein. These mutants might however be effecting the formation of a stable secondary structure that is present only when SmpB is bound to the ribosome but not present in solution. Bulky side chains or charged amino acids that inhibit the formation of a secondary structure of the C-terminal extension, most likely an alpha-helix based on structural prediction(63), prevent SmpB protein from functioning properly in *trans*-translation. It is also likely that the bulky amino acids, or charged amino acids might interfere with contacts made by the Lys-131, Lys-133 and Lys-134 residues surrounding Gly-132. Disruption of these contacts would lead to a decrease in the ability of SmpB to support the tagging function of *trans*-translation (See Following).

An intriguing observation made while analyzing the multiple sequence alignment of SmpB protein was that Gly-132 was surrounded by charged highly conserved amino

acids. I hypothesized that not only might the Gly-132 residue be playing an important role in tmRNA-SmpB mediated *trans*-translation, the whole region might be playing an important role. To investigate this possibility I made single, double and triple alanine substitution variants to analyze their contribution to tmRNA-SmpB mediated *trans*-translation. Interestingly, the doubly and triply substituted SmpB variants had a deleterious affect on the ability of SmpB to tag endogenous proteins. The greater than 75% reduction in tagging displayed by the $SmpB^{K131A/K133A/K134A}$ variant is in stark contrast to the $SmpB^{N17A/K18A/R19A}$ variant, that despite being a highly conserved region of SmpB, is fully functional in the ability to support the tagging function of *trans*-translation (Figure 4.5). This result supports our hypothesis that the Lys-131 to Lys-K134 region of SmpB is playing a critical role in tmRNA-SmpB mediated *trans*-translation.

The highly conserved nature of the Lys-131, Lys-133 and Lys-134 residues and their spatial positioning on SmpB, has led to speculation in the literature as to the role these amino acids play on SmpB protein(55, 61). Originally thought of as part of an RNA binding surface, it is clear from Figure 4.7 that these amino acids are not playing a role in the ability of SmpB to bind to the tRNA-like domain of tmRNA(55). This result is not surprising being that the SmpB Lys-131 to Lys-134 amino acids are located on a face of SmpB protein opposite that of the SmpB-tmRNA binding residues (Figure 4.2B). More recently, it has been speculated that these amino acids are playing a critical role in the ability of SmpB to bind to stalled ribosomes. Surprisingly though, these amino acids are not playing a critical role in the ability of SmpB to bind to and stably associate with stalled ribosomes. As shown in Figure 4.6A by Western blotting, $SmpB^{K131A/K133A/K134A}$ is

fully capable of associating with stalled ribosomes. Northern blotting also reveals that $SmpB^{K131A/K133A/K134A}$ is capable of delivering tmRNA to stalled ribosomes (Figure 4.6B).

These results leave a number of unanswered questions as to the specific role that the various conserved amino acids are playing in *trans*-translation. Firstly, mutations to the highly conserved residues Asp-17, Lys-18 and Arg-19 do not have an affect on SmpB protein that is detectable in any of the assays I have utilized to analyze the SmpB variants. In fact, deletion of the first 23 amino acids of SmpB does not give a significant tagging phenotype (AWK unpublished observations). Although these amino acids do not appear to have a role in a function of SmpB protein that would affectthe tagging function, this region of SmpB might play a role in an auxiliary function performed by SmpB protein. It is entirely possible that these SmpB amino acids are involved in some capacity in the degradation of aberrant mRNAs, possibly by delivering a component of the RNA degradation machinery to the ribosome. It is also possible that these amino acids may play a role in the degradation of tmRNA-tagged proteins by facilitating the delivery of a tmRNA-tag-specific protease or chaperone protein such as SspB to the ribosome. If the N-terminal region of SmpB (and specifically the SmpB Asp-17 to Arg-19 region) provide a function to enable either RNA or protein degradation, our methods of analysis would be unable to elucidate them.

My analysis of the Lys-131 to Lys-134 region of SmpB reveals that this region of SmpB protein is critically important for the function of SmpB in *trans*-translation. This region of SmpB protein does not play a role in binding to the tRNA-like domain of tmRNA and does not play a critical role in tmRNA-SmpB-ribosome association. Insight gained from the substitution of SmpB Gly-132 reveals that there appears to be a

requirement for small amino acids at this position and that glycine is ideally suited in *E.coli* to perform at this position. This requirement is mirrored by the multiple sequence alignment that shows that glycine is 98% conserved at this position while only being replaced by proline in *Mycoplasma*. Interestingly though, substitution of Gly-132 with proline in *E.coli* was only able to support the tagging function to 60% of WT levels (Fig. 4.3B).

The conserved lysine residues surrounding Gly-132 have been predicted to comprise an RNA binding surface(55). The data presented here does not demonstrate that these amino acids are not making contacts with rRNA, only that they are not critical for tmRNA-SmpB-ribosome association. In fact, the Lys-133 residue has recently been shown by hydroxyl-radical probing to be tantalizingly close to important rRNA nucleotides within the ribosomal A-site(142). Perhaps the Lys-131 to Lys-134 region is providing critical contacts, within the context of the ribosome, that are critical for the tagging function of SmpB. The phenotype of the SmpB Lys-131 to Lys-134 variants is similar to the phenotype of the C-terminal tail truncation and substitution variants reported on previously(63, 64). It is possible that the Lys-131 to Lys-134 region works in concert with the rest of the C-terminal tail to elicit trans-peptidation by acting as an anti-codon mimic. Consistent with this hypothesis, the Lys-131 to Lys-134 region projects from the core structure of SmpB and may play a role in positioning of the C-terminal tail within the ribosome allowing the distal part of the C-terminal tail to act as an anti-codon mimic.

It is likely that recognition of stalled ribosomes by the SmpB-tmRNA-EF-Tu ribonucleoprotein complex is a multipartite interaction, comprising SmpB residues,

tmRNA nucleotides as well as residues located on EF-Tu. I feel that this is the most likely means by which stalled ribosomes are recognized. If this is indeed the case, I cannot exclude the SmpB Lys-131 to Lys-134 region from this interaction. However, data presented here suggests that these interactions are not critical for recognition of stalled ribosomes.

This study sheds light on the roles played by SmpB in *trans*-translation. I have identified amino acids of SmpB protein that are critical for SmpB function in *trans*-translation. I have shown that these amino acids are not involved in either tmRNA binding and nor do they play a critical role in tmRNA-SmpB-ribosome association. It is clear that these amino acids are playing an important role in an SmpB function independent of tmRNA binding or stalled ribosome recognition. Hydroxyl-radical probing data positions these amino acids within the ribosomal A-site and these residues might provide critical contacts that are necessary for accommodation of tmRNA into the ribosomal A-site and transpeptidation.

4.6 Figures

Figure 4.1:

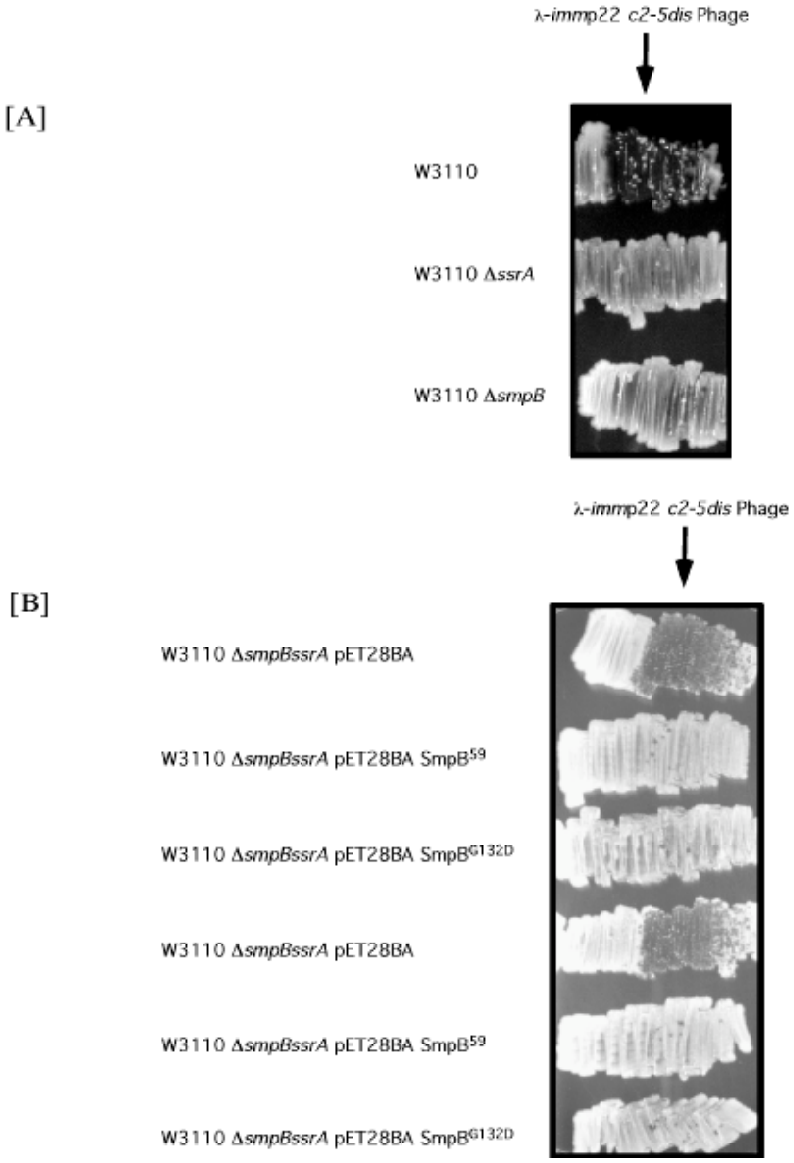


Figure 4.1: λ*imm*p22 *c2-5dis* hybrid phage cross streak assays. (A) Phage was spotted onto LB plates and W3110, W3110*ΔssrA*, and W3110*ΔsmpB* were streaked through the phage spot. (B) λ*imm*p22 *c2-5dis* hybrid phage cross streak assay analyzing the ability of SmpB variants $SmpB^{59}$ and $SmpB^{G132D}$ to support lytic induction. Phage assays in (B) are shown in duplicate.

Figure 4.2:

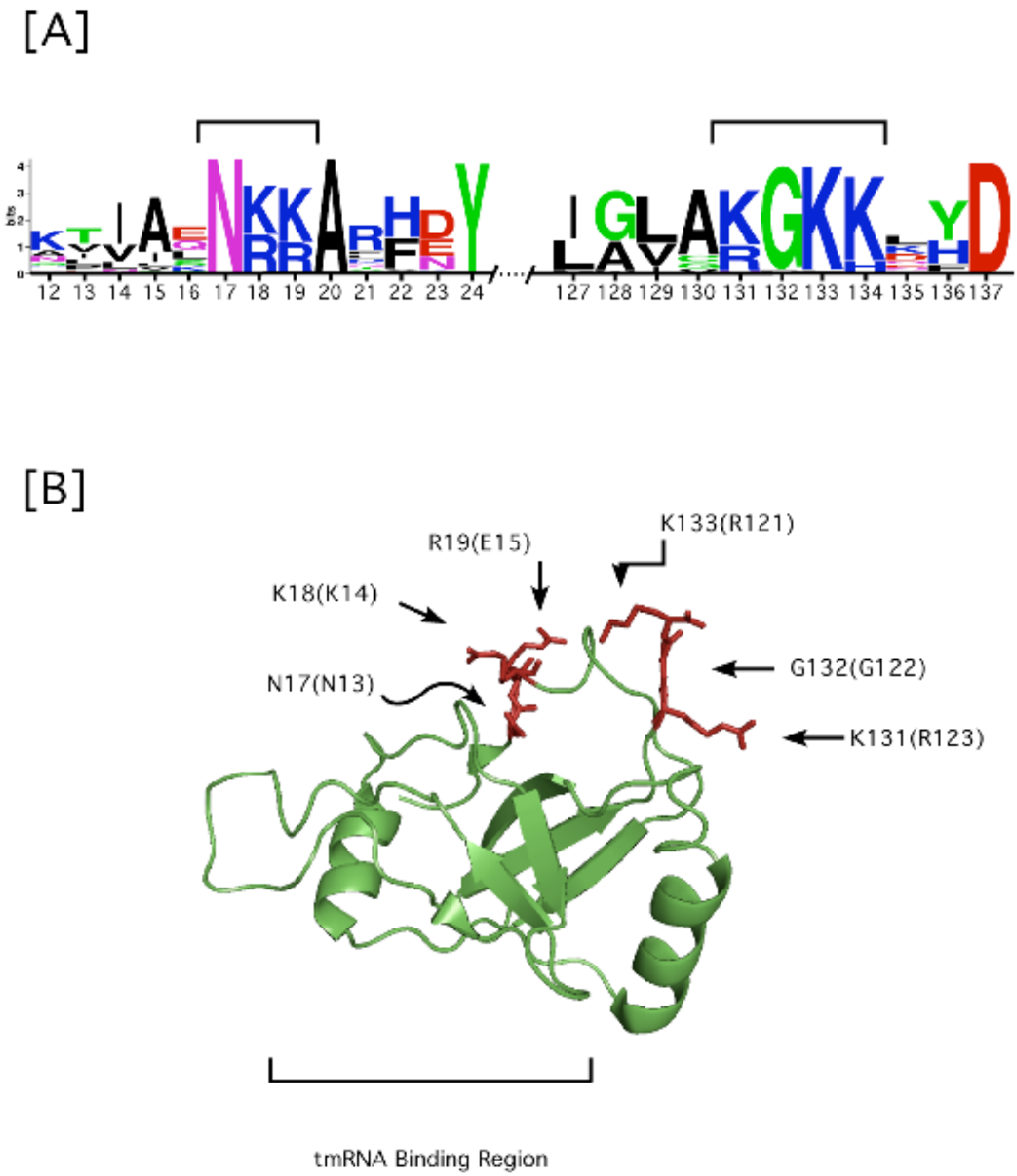


Figure 4.2: (A) Web logo representation of a multiple sequence alignment of SmpB protein. Brackets indicate the regions of SmpB investigated in this study. (B) Ribbon drawing of the tertiary structure of wild-type *T. Thermophilus* SmpB. The side-chains of residues analyzed in this study are shown in a ball-and-stick representation. The tmRNA binding surface of SmpB is indicated by a bracket. The Figure was prepared using the PDB file 1WJX, Yokoyama et al, and Pymol.

Figure 4.3:

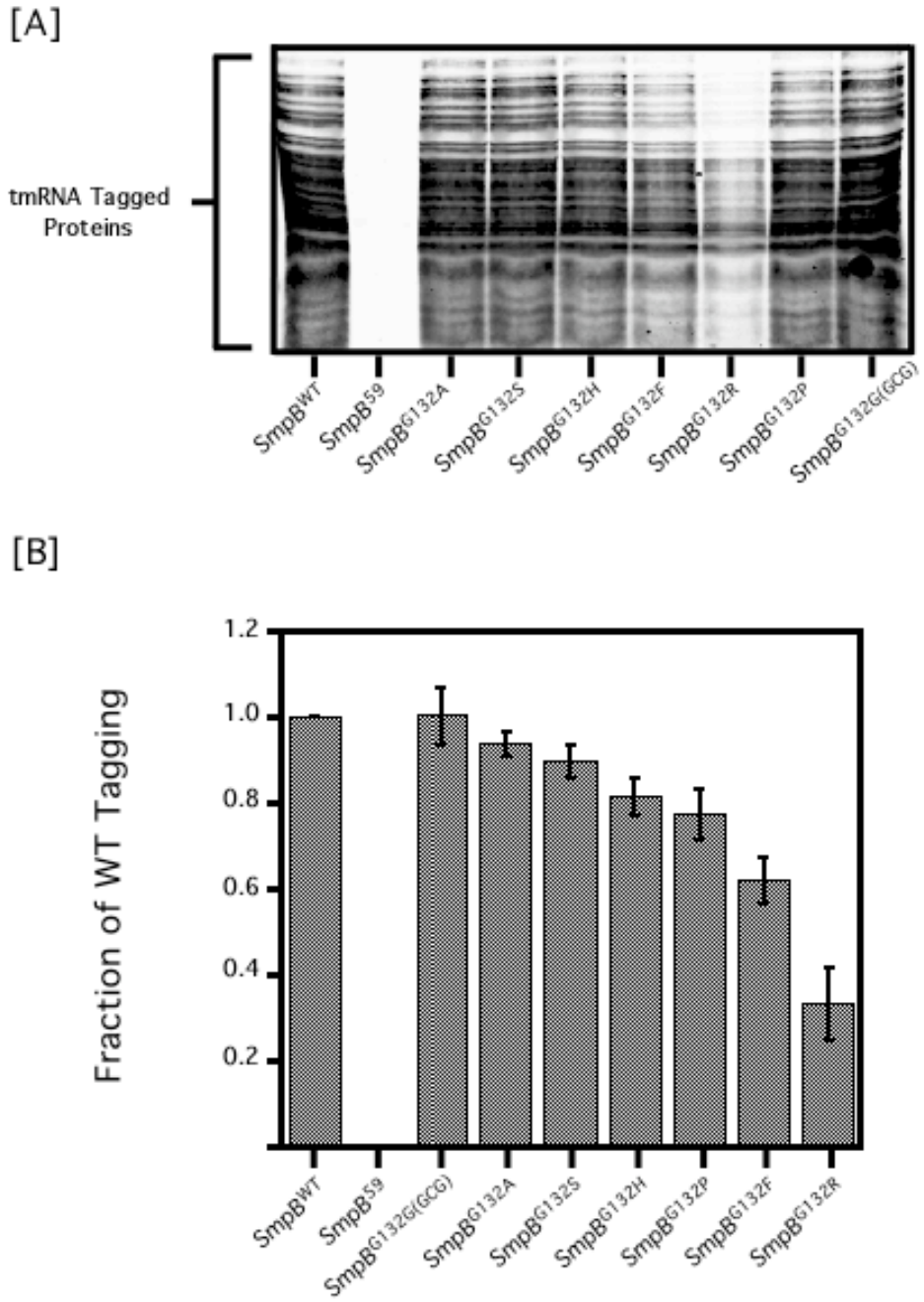


Figure 4.3: Analysis of the endogenous tagging activity of *E. coli* SmpB^{G132X} complementing clones (A) A representative Western blot, developed with antibodies to the 6-His epitope, displaying all of the endogenously tagged proteins. (B) Bar graphs depicting the mean and standard deviation of 3 independent tagging assays displaying the level of tagging activity relative to wild-type SmpB protein. SmpB variants in (B) are ordered from left to right in order of decreasing tagging proficiency.

Figure 4.4:

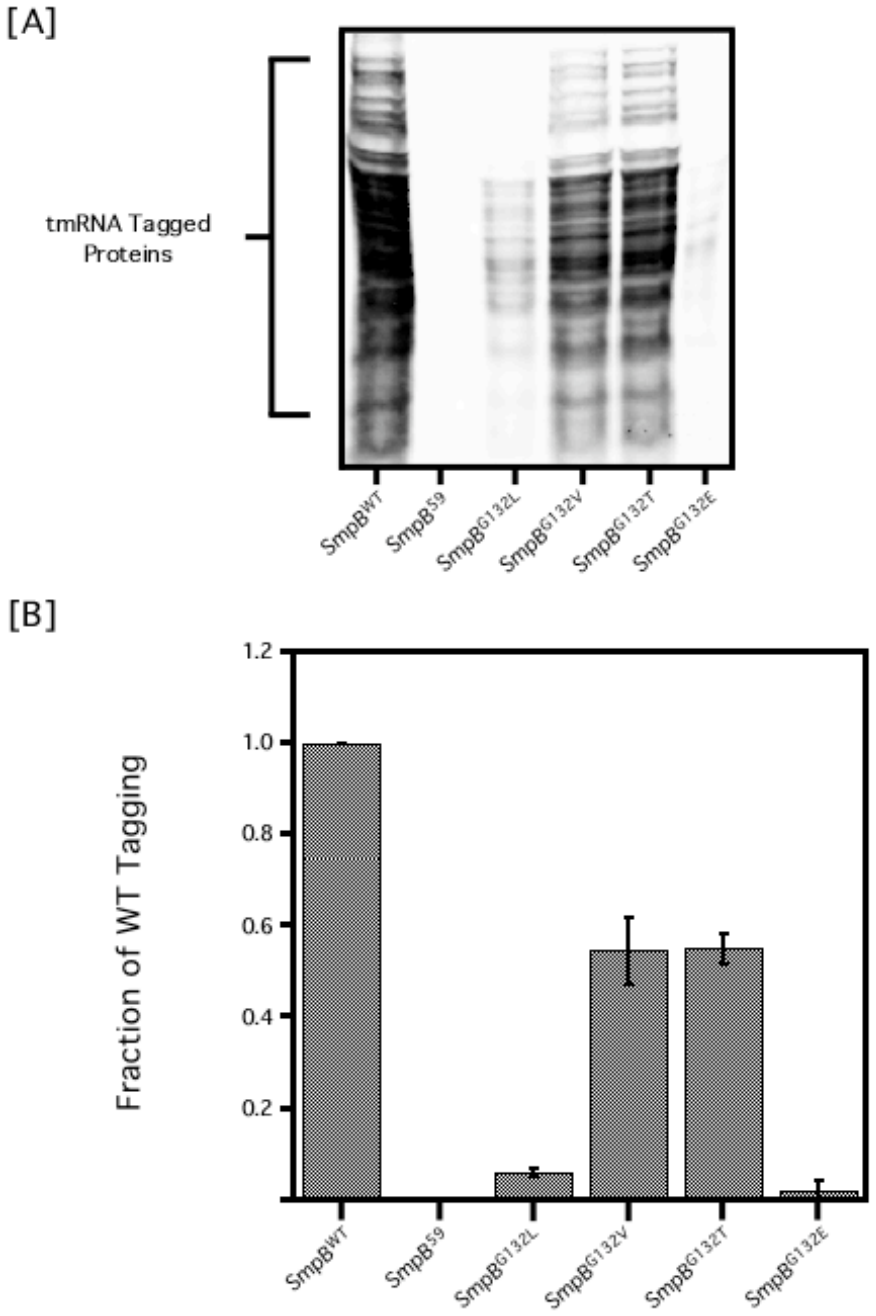


Figure 4.4: Analysis of the endogenous tagging activity of *E. coli* SmpB^{G132X} non-complementing clones (A) A representative Western blot, developed with antibodies to the 6-His epitope, displaying all of the endogenously tagged proteins. (B) Bar graphs depicting the mean and standard deviation of 3 independent tagging assays displaying the level of tagging activity as compared to wild-type SmpB protein.

Figure 4.5:

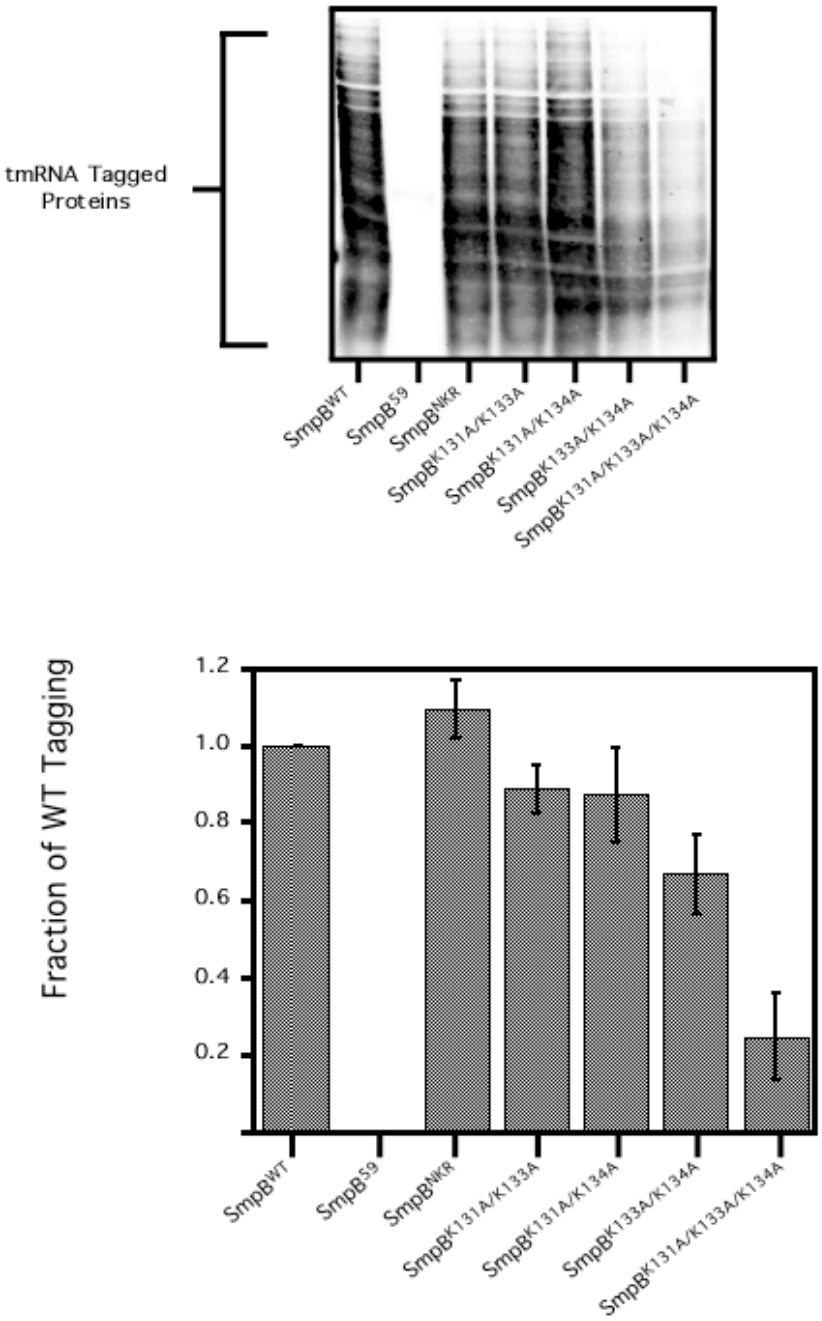


Figure 4.5: Analysis of the endogenous tagging activity of *E. coli* SmpB alanine substitution variants in the Asn-17 to Arg-19 region and the Lys-131 to Lys-134 region of SmpB (A) A representative Western blot, developed with antibodies to the 6-His epitope, displaying all of the endogenously tagged proteins. $SmpB^{N17A/K18A/R19A}$ variant is represented as $SmpB^{NKR}$. (B) Bar graphs depicting the mean and standard deviation of 3 independent tagging assays displaying the level of tagging activity as compared to wild-type SmpB protein.

Figure 4.6:

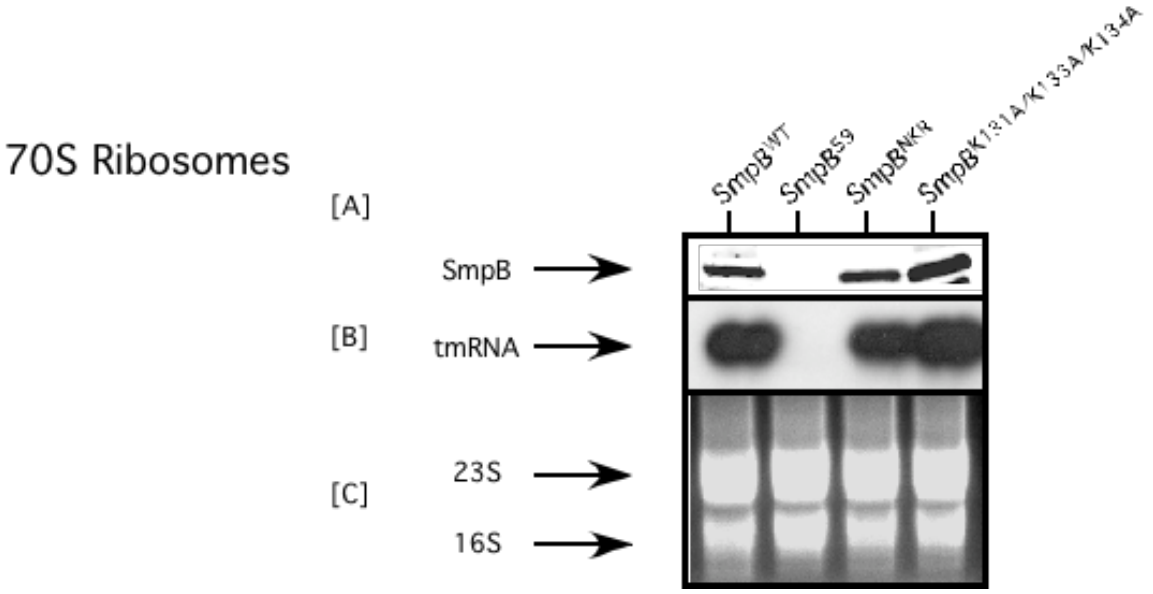


Figure 4.6: Ribosome association assays. (A) Western blot analysis with anti-SmpB antibodies displaying the amount of wild-type SmpB with select SmpB alanine substitution variants, associated with 70S ribosomes *in vivo*. (B) Northern blot analysis with a tmRNA specific probe to detect 70S ribosome associated tmRNA. (C) Ethidium bromide staining of the same gel as in *B,* shown to demonstrate that similar amounts of ribosomal RNA were loaded in each lane. The SmpB variant expressed in the cells from which the ribosomes were purified is indicated on top of each panel. $SmpB^{N17AK18A/R19A}$ is represented as $SmpB^{NKR}$. Wild-type SmpB is used as a positive control and $SmpB^{59}$, a nonfunctional truncated SmpB variant, is used as a negative control.

Figure 4.7:

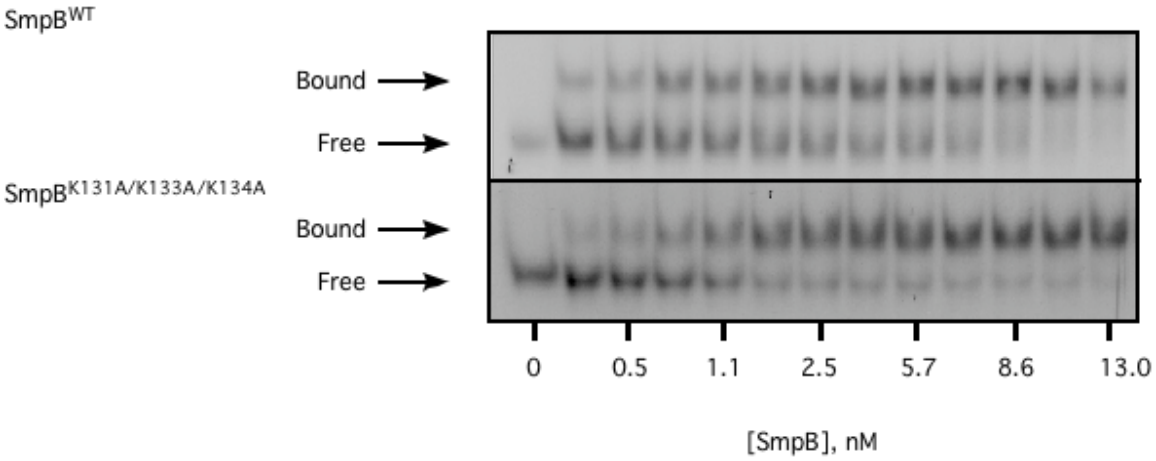


Figure 4.7: Gel mobility-shift assays of the binding propensities of $SmpB^{WT}$, and $SmpB^{K131A/K133A/K134A}$ to *E. coli* $tmRNA^{113}$ RNA. The position of free and SmpB bound $tmRNA^{113}$ is indicated by arrows to the left of each panel.

Chapter 5: Concluding Remarks

5.1 Summary

This dissertation has provided new insights into the dynamic roles played by SmpB in *trans*-translation. Specifically, I have identified conserved SmpB amino acids critical for the function of SmpB and I have characterized the function of these amino acids in *trans*-translation. To fulfill the goal of this project, I used multiple approaches to identify key SmpB amino acids. In Chapter 3 I used site directed mutagenesis mutagenesis of the *E. coli* SmpB protein to identify residues critical for the function of SmpB. I determined that mutation of specific highly conserved residues (E31, L91, K124) led to a marked decrease in the ability of SmpB to support the tagging function of *trans*-translation. I described how mutation of these residues leads to a decrease in the ability of the SmpB-tmRNA complex to associate with ribosomes. I determined that the decrease in the ability of the SmpB-tmRNA complex to associate with ribosomes was due to a decrease in the binding affinity of SmpB for tmRNA. This decrease was due to loss of specific contacts between SmpB amino acids and the tRNA-like domain of tmRNA. This work gives us key insights into how SmpB binds to tmRNA and the energetic contributions provided by specific amino acids of SmpB.

The amino acids that I identified to be critical for *E. coli* SmpB-tmRNA interactions are highly conserved in the primary amino acid sequence of SmpB throughout bacteria. I present evidence that these residues have been conserved for tmRNA binding. Mutations to the corresponding residues in *Thermoanerobacter*

tengcongensis(Tten) SmpB led to a significant decrease in the affinity of Tten SmpB for Tten tmRNA. Taken together it has now become clear that these residues represent conserved RNA binding contacts that are critical for SmpB-tmRNA binding. This work also demonstrates the need for biochemical data to support crystallographic data. Coincidentally, while this work was being undertaken, another group published a co-crystal structure of the tRNA-like domain of tmRNA bound to SmpB(40). Although the co-crystal structure depicted the tRNA-like domain binding to the oligonucleotide-binding fold of SmpB, the specific contacts being made were unresolved. My work has convincingly determined what residues are involved in tmRNA binding and quantified their contribution. My work also highlights the consequences of losing SmpB-tmRNA contacts *in vivo*, namely loss of ribosome binding resulting in a decrease in endogenously tagged proteins. Recently a higher resolution co-crystal structure of SmpB bound to the tRNA-like domain of tmRNA has become available and my results are bolstered by the higher resolution structure(41).

I have also presented multiple lines of evidence for an important role of the SmpB C-terminal tail in *trans*-translation. In separate investigations I identified critical SmpB amino acids within the C-terminal tail and show that they must be involved in a novel function of SmpB protein beyond tmRNA binding and stable association with 70S ribosomes. In Chapter 2, using multiple sequence alignment data I identified SmpB residues D137, K138 and R139 as being highly conserved. I went on to show that mutation of these amino acids leads to a defect in the ability of SmpB to support the tagging function of *trans*-translation. However, these SmpB mutants are fully proficient in the ability to bind tmRNA and deliver tmRNA to the ribosome. These mutants have a

similar phenotype to SmpB C-terminal tail deletion mutants, defective in tagging but fully proficient in tmRNA binding and ribosome association. These C-terminal tail mutants fail to add the tmRNA alanine charge and it is likely that the SmpB D137/K138/R139 mutants have a similar defect.

I have also identified SmpB amino acids located within the proximal region of the SmpB C-terminal tail that are critical for the function of SmpB (Chapter 4). Although it had been speculated that these amino acids are critical for SmpB-ribosome binding, it now appears clear that this is not the case. My data suggests a role for these SmpB amino acids (K131 to K134) similar to the role afforded to the rest of the C-terminal tail. In fact, this region of SmpB (K131 to K134) might act as an anchor for the more distal regions of the tail (possibly D137 to R139) and allow positioning of the distal regions of the tail within the ribosomal A-site.

5.2 Remaining Questions

Is there a role for the SmpB N-terminal region?

Due to the conserved nature of a number of conserved amino acids within the SmpB N-terminal region I believe that it is likely that this region plays a role in *trans*-translation. Although I was unable to uncover this role, sequence conservation suggests that this region of SmpB is important. SmpB plays a central role in the *trans*-translation mechanism and is required for ribosome rescue, targeted degradation of the aberrant mRNAs as well as delivering tmRNA to the ribosome to tag incomplete proteins. SmpB might deliver a factor, outside of tmRNA, to the ribosome to direct one of these other

functions, mRNA or protein degradation. To test for a role in mRNA degradation a mRNA lacking a stop codon could be expressed in a strain harboring an SmpB N-terminal mutant and the stability of this message could be compared to an SmpB wild-type strain. Similarly, if one had antibodies to the tmRNA degradation tag that is appended to incomplete proteins following ribosome stalling, one could assay for the stability of tagged protein products in a strain harboring an SmpB N-terminal mutant and the stability of tagged protein products could be compared to a wild-type SmpB strain.

What specific role does the SmpB C-terminal tail play in accommodation?

My investigation of residues critical for the function of SmpB uncovered multiple residues within the SmpB C-terminal tail that are required for a novel function of SmpB. A next logical step would to be to determine what function the SmpB C-terminal tail is performing. Due to the complexity of these interactions it will undoubtedly take multiple experimental approaches to characterize this novel role. One line of experiments that would shed light on what role these amino acids are playing could be gleaned by SmpB-rRNA cross linking experiments to determine what rRNA nucleotides SmpB might be interacting with. Along those lines, footprinting or hydroxyl radical probing of the ribosome bound by the tmRNA-SmpB complex using a series of SmpB C-terminal tail variants might elucidate the molecular contacts between SmpB and rRNA. One report using hydroxyl radicals determined that the SmpB C-terminal residue K133 is in the vicinity of the 30S ribosomal A-site (16S rRNA residue 1398(62)) but unfortunately they did not investigate the D137 to R139 region. More experimental evidence in this area

would help to determine the position of SmpB on the ribosome. Ideally, the crystal structure of the SmpB-tmRNA complex bound to 70S ribosomes at a distinct stages of *trans*-translation would yield insight into the role of the SmpB C-terminal tail, as well as other regions of tmRNA.

It is also entirely possible that the proximal region of the SmpB C-terminal tail is interacting with the ribosome while the distal region of the tail, in the context of the ribosome, contacts a region of tmRNA outside of the tRNA-like domain. One interesting hypothesis is that within the ribosome the SmpB D137/K138/R139 region might be acting as a tripeptide 'anticodon' in a manner similar to release factors (20, 143) and binding to a region of tmRNA to allow full accommodation to occur. Work in this area will not only shed light on the mechanism of *trans*-translation but will also reveal some of the dynamic roles played by the ribosome during translation.

How does the SmpB-tmRNA complex recognize stalled ribosomes?

This is another key question in the field of *trans*-translation that has yet to be answered. I had hoped that during my investigations of SmpB-tmRNA interactions I would have uncovered residues that are critical for the interaction of this complex with ribosomes that are unrelated to tmRNA binding. Unfortunately, my efforts have not yielded great insights into this area. I feel that due to the fact that a large multicompnent ribonucleoprotein complex containing SmpB-tmRNA and EF-Tu is recognizing stalled ribosomes, it is likely that contacts made by all three components are involved in stalled ribosome recognition. Similar experiments to those listed above (hydroxyl radical

probing, footprinting etc.) would help to elucidate the contacts between the stalled ribosome recognition complex and stalled ribosomes.

Table 5.1: Table of SmpB variants analyzed in this thesis as well as some not described. Tagging activity is compared to wild-type SmpB and (~) denotes an approximate value. Equilibrium dissociation constants (Kd) were determined as in (54, 64, 144) by curve fit analysis. Where curve fit analysis was not performed (~) denotes an approximate value for the Kd determined by visual analysis at the concentration of SmpB where 50% of tmRNA is bound. The λ*imm*p22 *c2-5dis* hybrid phage cross streak assay sensitivity is listed as R-(resistance to lysis), S-(sensitive to phage lysis), or MS-(moderately sensitive to phage lysis) as determined by visual inspection of cross streak assay. SmpB variants not functionally analyzed are listed as not determined (ND).

SmpB Variant	Tagging Activity (% Wild-type or estimation of % wild-type)	SmpB-tmRNA Binding Affinity (nM)	λ*imm*p22 *c2-5dis* hybrid phage cross streak assay R-Resistant to phage lysis S-Sensitive to phage lysis MS-Moderately Sensitive to phage lysis
Escherichia coli			
SmpB[59]	0	ND	R
SmpB$^{\Delta 20}$	~85	ND	ND
SmpB$^{\Delta 21}$	~80	ND	ND
N17A	~100	ND	ND
K18A	~100	ND	ND
R19A	~100	ND	ND
R19E	ND	ND	ND
N17A/K18A/R19A	100	~1nM	ND
K18E/R19E	~100	ND	MS
N17A/K18E/R19E	~100	ND	ND
A20P	~100	ND	ND
Y24E	~60	~65-100	MS
Y24E/Y55E	0	>500	ND
E31A	77	29+/-3	ND
E31A/L91A	77	31+/-4	ND
E31A/K124A	36	140+/-5	ND
E31A/L91A/K124A	30	174+/-3	ND
R45A	100	ND	ND
R45A/W118A	100	ND	ND
Y55E	~20	ND	ND
R86E	~85	ND	MS
L91A	87	58+/-5	ND
L91A/K124A	52	87+/-5	ND
N93A	112	26+/-2	ND
N93H	ND	~0.7	ND
Q94A	105	7+/-2	ND
Q94K	ND	~0.5	ND

N93A/Q94A	111	29+/-3	ND
N93H/Q94K	ND	~0.3	ND
N93H/Q94K/W122K	ND	~0.8	ND
N93H/Q94K/W122R	ND	ND	ND
W118A	95		ND
W122A	ND	~20-30	ND
K124A	87	49+/-3	ND
K131A	~100	ND	ND
K131E	107	ND	ND
K131A/K133A	89	ND	ND
K131A/K134A	87	ND	ND
K131A/K133A/K134A	24	~1	ND
G132A	94	ND	ND
G132D	ND	~1	R
G132E	2	ND	MS
G132F	62	ND	MS
G132H	82	ND	R
G132L	6	ND	MS
G132P	77	ND	MS
G132R	33	ND	S
G132S	89	ND	R
G132T	55	ND	R
G132V	54	ND	ND
K133A	~100	ND	ND
K133E	111	ND	ND
K133A/K134A	67	ND	ND
K133E/K134E	73	ND	ND
K134A	~100	ND	ND
K134E	100	ND	ND
D137A	~100	ND	ND
D137R	154	ND	ND
D137A/R139A	178	ND	ND
D137A/K138A/R139A	13	0.68+/-0.7	ND
D137R/R139E	182	ND	ND
K138A	~100	ND	ND
K138E	134	ND	ND
K138A/R139A	42	0.87+/-0.14	ND
R139A	~90	ND	ND
R139E	48	0.41+/-0.05	ND
Thermoanaerobacter tengcongensis SmpB			
			N/A
Wild-type	N/A	12+/-2	N/A
E28A	N/A	120+/-10	N/A

L87A	N/A	350+/-40	N/A
H89A	N/A	290+/-18	N/A
R90A	N/A	50+/-6	N/A
K120A	N/A	550+/-50	

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