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Identification and Characterization of Lon Protease as a Component of Bacterial *trans*-Translation

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Abstract of the Dissertation

Identification and Characterization of Lon Protease as a Component of Bacterial *trans*-Translation

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The *trans*-translation pathway of bacteria serves to counteract problems associated with interrupted protein synthesis. During *trans*-translation, a trapped ribosomal complex that is unable to continue or terminate is properly released, the associated mRNA is rapidly degraded, and the incomplete peptide is tagged for targeted proteolysis. Ribosomal rescue helps maintain the pool of functional ribosomes, degradation of the ribosome-stalling mRNA prevents the recurrence of interrupted translation, and degradation of the incomplete peptide limits the cellular burden of abnormal proteins. The bifunctional transfer-messenger RNA (tmRNA) and its dedicated cofactor SmpB protein are essential components of *trans*-translation. Genetic screens were designed to identify novel components of *trans*-translation and further characterize this highly conserved pathway. 18,929 *Escherichia coli* mutants generated by transposon mutagenesis were screened

for a specific bacteriophage phenotype associated with cells defective in tmRNA and SmpB, producing 148 primary candidates. Colony PCR analyses of the segment of *E. coli* genomic DNA containing the genes encoding tmRNA and SmpB suggested that two of the primary candidates contained transposon in this region. To remove mutants that were generally resistant to bacteriophage infection, the primary candidates were screened for sensitivity to a bacteriophage whose development is independent of tmRNA and SmpB function. The secondary bacteriophage screening of the primary candidates left 16 secondary candidates, which were mapped to determine transposon integration sites. Assessment of *trans*-translation function in the secondary candidates using an endogenous protein tagging assay revealed that each of the three candidates with transposon integrated in the gene encoding Lon protease accumulated excessive levels of the tagged proteins produced during *trans*-translation. Two reporter assays were optimized specifically for the study of tagged protein turnover *in vivo* and confirmed that cells defective in Lon protease are unable to efficiently dispose of tagged peptides compared to wild-type cells. *In vitro* proteolysis experiments using highly purified components showed that Lon preferentially degrades tagged proteins compared to untagged control proteins, thus complementing *in vivo* experiments. This dissertation discusses the use of genetic screens for the investigation of *trans*-translation and the experimental course used for the characterization a strong screen candidate.

This dissertation is dedicated to Troy and Gabrielle Parla.

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Publications

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Dulebohn, D., **Choy, J.,** Sundermeier, T., Okan, N., and Karzai, A.W. 2007. *Trans*-Translation: The tmRNA-Mediated Surveillance Mechanism for Ribosome Rescue, Directed Protein Degradation, and Nonstop mRNA Decay. *Biochemistry*. **46**(16):4681-93.

Chapter 1: Introduction

The conversion of genetic information to actual cellular factors that function in key processes that serve to sustain and perpetuate life is a cornerstone of biology. One of the major cellular processes involved in the derivation of information stored in genes is translation. Accordingly, the mechanistic details and the major components of translation are highly conserved throughout the three domains of life: Bacteria, Archaea, and Eukarya (24, 67, 122, 124, 193). However, there are important differences in translation among these three kingdoms, and it appears that archaea are more similar to eukaryotes than to bacteria in this regard (91, 112, 117). Further insights into the details of translation in each of the three kingdoms should prove to be very interesting in describing the ways an essential biological mechanism can differ during the course of evolution.

The relative ease of handling bacteria in the laboratory setting and the high conservation of translation across life-forms make bacteria an invaluable source of information for researchers interested in the mechanistic details of protein synthesis. Furthermore, with the societal burden of numerous pathogenic bacterial species, the distinguishing characteristics of bacterial translation can be studied and exploited to our advantage.

Bacterial Translation

Translation is a complex and intricate process that involves an array of RNAs and supporting proteins (147). As a mechanism of gene decoding, translation is connected to RNA transcription by its requirement for messenger RNA (mRNA) templates from which proteins are built. Typically, a translation-competent mRNA contains a ribosome binding site, or Shine-Dalgarno sequence, in its 5'-untranslated region, a start codon that is most commonly AUG, a coding region, and one of the three termination codons UAA, UAG, and UGA. Thus, in addition to carrying protein-coding information, mRNAs must also carry directing information for the translation process. The collection of cellular components that are necessary for protein synthesis additionally include ribosomes, transfer RNAs (tRNAs), aminoacyl-tRNA synthetases, and a group of dedicated proteins called translation factors.

The 70S bacterial ribosome is a large complex of ribosomal RNA (rRNA) and protein, formed from the association of the smaller 30S ribosomal subunit and the larger 50S ribosomal subunit (147). Each ribosome contains three functionally distinct tRNA binding sites that span both subunits. The aminoacyl (A) site accepts the incoming aminoacyl-tRNA (aa-tRNA), the peptidyl (P) site contains the tRNA carrying the nascent peptide chain, and the exit (E) site carries the deacylated tRNA following the transfer of the nascent peptide (202). Each ribosomal subunit contains additional functional regions crucial for the proper execution of translation (147). The 30S subunit is responsible for binding

the mRNA, which is a critical step of initiation, and contributes to translation fidelity by ensuring that the correct aa-tRNA base-pairs with the mRNA codon in the ribosomal A site. The 50S subunit contains the GTPase-associated center, which is essential for promoting GTP hydrolysis on the G protein translation factors that help drive translation, and the peptidyl transferase center, which catalyzes peptide bond formation.

Each cycle of translation is characterized by three phases, namely initiation, elongation, and termination. During translation initiation, the 30S and 50S ribosomal subunits assemble around the start codon of an mRNA in a cooperative manner that requires initiation factors IF3, IF1, and IF2, GTP, and the initiator tRNA fMet-tRNA^{fMet}, forming the 70S initiation complex (43). Translation initiation is a streamlined event that follows from the termination of a previous round of translation by the association of IF3 with the 30S subunit at both the beginning and the end of each cycle of translation (28, 62, 93, 160). The completion of initiation is marked by the dissociation of the initiation factors from the 70S initiation complex as a result of GTP hydrolysis on IF2 (118, 155). The resulting ribosome contains fMet-tRNA^{fMet} in its P site and an empty A site, and is ready for elongation.

The critical steps of elongation (147) include mRNA decoding, peptide bond formation, and translocation and employ the functions of elongation factors (EFs). During elongation, the ribosome catalyzes the synthesis of protein from mRNA code in a carefully controlled stepwise manner that maintains the reading frame. Aminoacyl-tRNAs are individually delivered to the ribosome as part of EF-

Tu•GTP•aa-tRNA ternary complexes. Correct base pairing between the anticodon stem of the aa-tRNA and the A site codon is followed by GTP hydrolysis on EF-Tu and A site accommodation of the cognate aa-tRNA (16, 138, 139). Accommodation is immediately followed by peptidyl transferase activity in the 50S subunit, resulting in the addition of the A site tRNA amino acid onto the nascent peptide (139). Once peptide bond synthesis has occurred, the ribosome carries a deacylated tRNA in its P site and a peptidyl-tRNA in its A site. In order to continue elongation, translocation is required to move the tRNAs, from P site to E site and from A site to P site, and to place the next mRNA codon in the A site. Translocation is driven forward by GTP hydrolysis on elongation factor EF-G (154).

Termination begins with a stop codon in the A site (147). Stop codons can be recognized by two release factors (RFs). Specifically, UAA is recognized by both RF1 and RF2, UAG is recognized by RF1 only, and UGA is recognized by RF2 only (38). Importantly, the efficiency of termination compared to non-termination events, such as frame-shifting and nonsense suppression, is influenced by stop codon context. mRNA nucleotides just downstream of the stop codon (120, 144) and the penultimate and ultimate residues of the nascent peptide charged to the P site tRNA (15, 130) are known to modulate termination efficiency. The mRNA footprint of RF2 was also found to be larger than that needed to span a UGA stop codon in the A site (145). The actions of RF1 and RF2 ensure that the nascent peptide is properly released from the P site tRNA and from the ribosome before the ribosome is disassembled. RF1/2 are released

from the ribosome through displacement by RF3 (53, 54, 204). GTP hydrolysis on RF3 results in its own dissociation from the ribosome. Ribosome disassembly requires ribosome recycling factor (RRF) and EF-G (86, 93, 143). IF3 is also critical as an anti-association factor during termination to allow proper ribosome recycling, and, presumably, this interaction also allows for efficient initiation of a subsequent round of translation following termination (93, 160).

Interrupted Translation

The ribosome and the mechanism of translation have evolved to be very efficient. However, ribosomes are not individually equipped for the correction of forthcoming barriers. As a consequence of being an absolutely essential process, the severe interruption of translation, as opposed to transient pausing, can result in a myriad of undesirable cellular effects that jeopardize the survival of the organism. The detail and intricacy of translation is illustrated by the large collection of antibiotics that specifically target various aspects of translation (119, 140, 166, 175).

Interruptions may also occur without exogenous components, resulting from errors or limitations that arise from within the cell. The deleterious effects of interrupted translation are particularly critical during elongation and termination, when the cell has already invested a significant amount of cellular resources into the process. The endogenous causes of interrupted translation are often associated with the nature of the mRNA. For example, translation-blocking

mRNAs may lack stop codons (98), contain rare codons (116, 150, 153), or contain inefficient stop codons (36, 68, 69, 152, 172). Nonstop mRNAs may result from premature termination of transcription or ribonucleolytic cleavage, while an mRNA codon is considered rare if its cognate aa-tRNA is poorly represented in the cellular aa-tRNA pool. Rare aa-tRNAs are easily rate-limiting compared to the more common aa-tRNAs, and the particular aa-tRNAs that are rare vary depending on the organism (4). Stop codon efficiency is influenced by stop codon identity and by surrounding nucleotides (120, 144) and ribosome-associated cellular components (15, 130).

Regardless of the specific cause, interrupted translation can be described by ribosomal stalling. Stalled ribosomes cannot terminate normally and may accumulate on their mRNA transcripts, a fate that has been confirmed *in vitro* (82). Additionally, ribosomal stalling is more dramatic in bacteria due to their use of polysomes, which are collections of ribosomes that sequentially and simultaneously translate a given mRNA transcript. It has been estimated that problematic ribosomal stalling events occur once in every 250 translation events (127). Alone, the accumulation of trapped ribosomes is detrimental to the cell since this population of ribosomes is unable to contribute to active translation. However, this problem is complicated by the incomplete nascent peptides that accumulate along with trapped ribosomes and may possess deleterious activities due to truncation.

Transfer-Messenger RNA and SmpB protein

Interrupted translation, if not quickly solved, leads to unproductively stalled ribosomes, which are recognized as ribosomes that are unable to complete translation. To efficiently rescue stalled ribosomes and mitigate the cellular burden posed by truncated proteins, bacteria have evolved a mechanism called *trans*-translation (98). *Trans*-translation requires the activities of a unique RNA (114) called transfer-messenger RNA (tmRNA; also known as SsrA RNA and 10Sa RNA) and its protein cofactor (95) small protein B (SmpB). Together, tmRNA and SmpB function to allow the proper termination and recycling of stalled ribosomes and to target the degradation of the associated aberrant mRNAs and incomplete peptides. The genes for tmRNA and SmpB, *ssrA* and *smpB*, respectively, are represented in all species of bacteria with available genomic sequence data. The conservation of *ssrA* and *smpB* likely also reflects the extent of conservation and the evolutionary significance of the *trans*-translation pathway.

tmRNA is a small, stable RNA with a unique structure that reflects dual functions (Fig. 1.1). tmRNA is transcribed from the *ssrA* gene as a 457 nucleotide premature form. Premature tmRNA must be subjected to ribonucleolytic processing in order to produce the 363 nucleotide mature form (30, 107). The secondary structure of tmRNA (49, 189) shows two functionally distinct domains. The 5'- and 3'- ends of the RNA form a tRNA-like domain (TLD) that bears striking resemblance to $tRNA^{Ala}$ (49, 107, 189). Indeed, the acceptor arm of the

tmRNA TLD contains the G-U base pair determinant present in the $tRNA^{Ala}$ acceptor stem and is efficiently charged with alanine by alanyl-tRNA synthetase (107). Additional tRNA structural similarities include a T arm and a modified D loop. In place of a standard tRNA anticodon stem-loop is a connector stem that bridges the tmRNA tRNA-like and mRNA-like domains. The mRNA-like domain (MLD) of tmRNA contains an open reading frame (ORF) that encodes a ten amino acid peptide and ends with an in-frame stop codon. The small peptide encoded by this ORF serves as a degradation tag that is recognized by various cellular proteases. While the specific peptide sequence encoded by tmRNA varies among bacterial species (63), studies carried out using *E. coli* tmRNA have confirmed the ability of the tmRNA peptide tag to directly stimulate targeted proteolysis by the Tsp, ClpXP, ClpAP, and FtsH proteases (60, 72, 98). The MLD is further separated from the TLD by several pseudoknots, which are RNA stem-loop structures held together by intrastrand base pairing. *E. coli* tmRNA has four pseudoknots, one that precedes the MLD and three that follow the MLD. The true physiological relevance of these pseudoknots is still unclear, but it appears that pseudoknot one is the most critical for tmRNA function (132, 133, 135, 136, 196). Further, it still remains to be confirmed whether pseudoknot one bears a functional or a merely structural role in tmRNA activity (178).

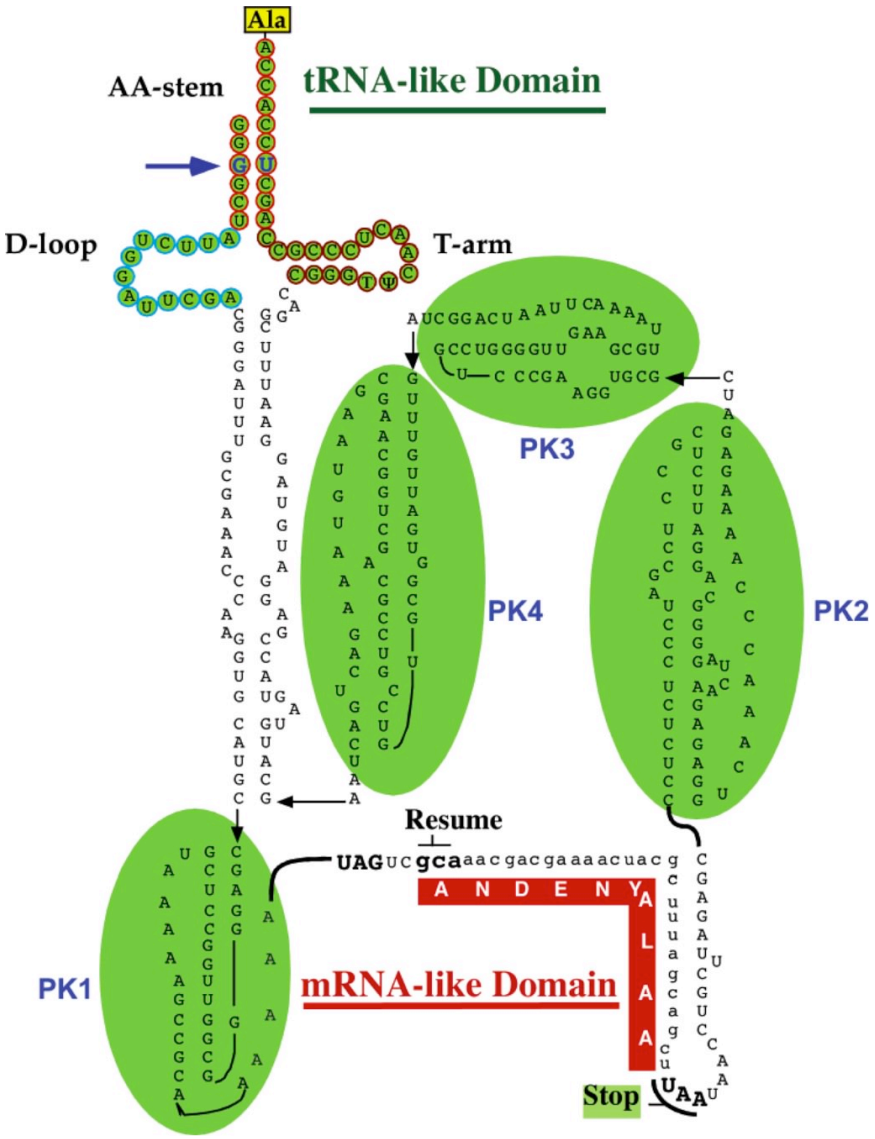

Fig. 1.1. Predicted secondary structure of *Escherichia coli* tmRNA. tmRNA is transcribed from the *ssrA* gene and is a bifunctional molecule with two distinct domains. The 5'- and 3'- ends of the mature tmRNA fold to form a tRNA-like domain (TLD) that contains a G-U base pair determinant (blue arrow) that is recognized by alanyl-tRNA synthetase for alanyl-charging of tmRNA. Instead of a standard tRNA anticodon stem-loop, tmRNA has a long connector stem that connects its TLD to its mRNA-like domain (MLD). The MLD of tmRNA encodes a ten amino acid degradation sequence and ends with an in-frame stop codon. Note that the alanyl-charge of tmRNA is not encoded by the MLD. Proteins that are tagged during *trans*-translation thus bear an eleven residue NH_2-AANDENYALAA-COOH tmRNA tag. SmpB protein binds to the TLD domain of tmRNA. Green ovals denote the four pseudoknots (PK1-4) of tmRNA. Figure from (44).

SmpB protein is a small, basic protein that has been shown to be absolutely required for tmRNA activity in *trans*-translation (95, 171). SmpB is essential for the stable association of alanyl-tmRNA with 70S ribosomes, but is not required for the maturation of tmRNA from its transcript precursor or for the aminoacylation of tmRNA (10, 11, 66, 95). The solution structures of SmpB confirmed that the protein consists of a central β-barrel that is surrounded by three peripheral α-helices (40, 165). Although the structure of SmpB is quite unique, an oligonucleotide-binding fold can be identified in its three-dimensional structure, supporting an RNA-binding function. The physical interaction of SmpB with tmRNA was initially shown using electrophoretic mobility shift assays (95) and has been confirmed using affinity chromatography, cryo-electron microscopy, and X-ray crystallography (64, 94, 183). The cocrystal structure of SmpB complexed with the TLD of tmRNA showed that a surface region of SmpB contacts the D loop and a short loop connecting the T arm and connector stem of tmRNA (64). The functional relevance of these SmpB•tmRNA contacts has been confirmed by biochemical studies (45, 66, 134). Notably, the very C-terminus of SmpB, consisting of approximately twenty residues, was disordered in the structural studies of the protein (40, 165). However, this region of SmpB has been shown to be critical for the activity of tmRNA in *trans*-translation (84, 171). SmpB C-terminal tail mutants bind tmRNA and support tmRNA association with ribosomes but do not enable the tRNA and mRNA functions of tmRNA (171).

Trans-Translation

The cellular substrate for *trans*-translation (Fig. 1.2) is an engaged ribosome that is unable to continue elongation or support termination. This ribosome is still complexed with mRNA, carries a peptidyl-tRNA in its P site, and presents a hallmark unoccupied A site. The A site is the point of ribosome entry for the SmpB•tmRNA complex, which is large compared to a standard aminoacyl-tRNA (64, 183). While the absence of an A site tRNA is critical for SmpB•tmRNA entry and accommodation, mRNA may still be present in the A site (6, 83). However, *in vitro* experiments have shown that there is a fifteen base limit for the length of mRNA that can extend past the P site in order to support the activation of *trans*-translation, with mRNAs carrying zero to six bases beyond the P site being preferred for the optimal activation of *trans*-translation (83). It is possible that in addition to reducing steric clashes that would hinder SmpB•tmRNA complex entry (183, 203), the restriction on downstream mRNA length (6, 83) influences the conformation of the ribosome around the A site in a manner that supports entry (128). Weakened or absent ribosome-mRNA contacts may allow a looser, more open conformation that supports SmpB•tmRNA entry (159, 203). Generally, the length of mRNA past the P site may vary depending on where the stalling event occurs on the mRNA and on the extent of stalling-dependent mRNA cleavage (70, 116, 153, 173, 174). The truncation mechanism for an mRNA with extensive sequence downstream of the ribosomal stall site is not well understood but is known to be stalling-dependent.

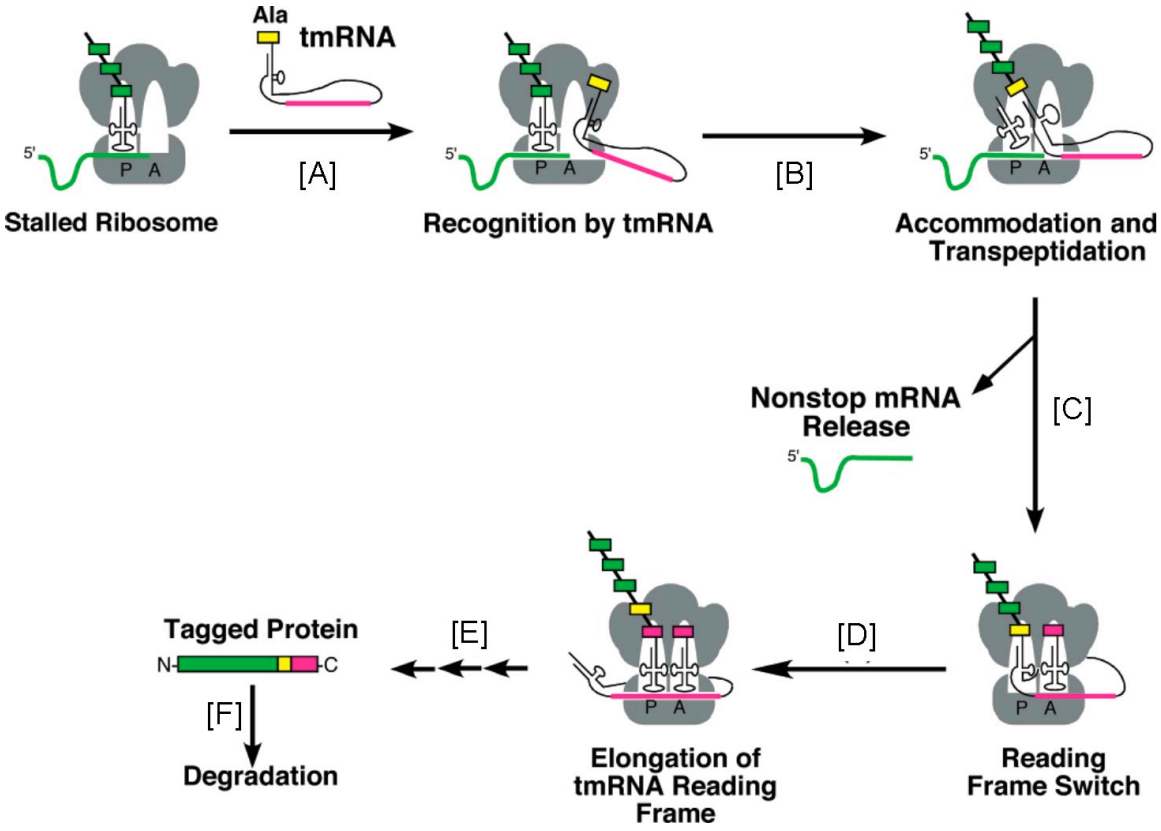

Fig. 1.2. Model of bacterial *trans*-translation. [A] *Trans*-translation is activated when a ribosome is unable to continue elongation or support proper termination. mRNAs that lack in-frame stop codons or carry rare codons are known activators of *trans*-translation. Stalled ribosomes with empty A sites are substrates for tmRNA recognition. [B] The accommodation of tmRNA into the A site of a stalled ribosome leads to peptidyl transfer of the nascent peptide onto the tRNA-like domain of tmRNA. [C] The ribosome cotranslationally switches from the faulty mRNA onto the mRNA-like domain of tmRNA. [D] Translation of the tmRNA open reading frame appends the tmRNA-encoded protein degradation tag onto the C-terminus of the nascent peptide. [E] Normal termination at the stop codon immediately following the tmRNA tag sequence supports ribosome rescue and recycling. [F] The released tmRNA-tagged protein is targeted for degradation by C-terminal specific cellular proteases. Figure adapted from (44).

A stalled ribosome with an unoccupied A site allows effective accommodation of the SmpB•tmRNA complex, which is delivered to the ribosome by EF-Tu•GTP in a manner analogous to the delivery of a cognate aa-tRNA and experiences little competition against cognate aa-tRNAs or release factors whose presence would have prevented ribosomal stalling in the first place (6, 36, 69, 83, 116, 153). Following the accommodation of the SmpB•tmRNA complex into the A site, a transpeptidation event occurs on the ribosome, whereby the nascent peptide carried by the P site tRNA is transferred onto the alanine charge on the tmRNA TLD. The exact mechanism of SmpB•tmRNA accommodation is unclear and must be non-canonical since tmRNA does not have an anticodon stem. Thus, peptidyl transfer onto tmRNA does not involve codon-anticodon decoding on the ribosome. Consistent with this, the alanine charge on tmRNA is not encoded in its MLD. Once the transpeptidation is complete, the ribosome is ready to switch from the stall-inducing mRNA onto the MLD of tmRNA. Translation resumes at a designated alanine codon in the tmRNA MLD and proceeds until the stop codon that marks the end of the tmRNA ORF. Translation terminates normally, allowing proper recycling of the ribosomal subunits and the release of a tagged protein. This chimeric protein consists of the peptide encoded by the aberrant mRNA C-terminally fused to the tmRNA degradation tag NH_2-AANDENYALAA-COOH.

Since the time when the *trans*-translation pathway was originally proposed (98), numerous studies have been undertaken to further clarify and elucidate its mechanistic details. Some key questions that were asked early on in the field

considered the fate of the faulty, ribosome-stalling mRNAs, the details of tmRNA and ribosome association, the ability of the ribosome to cotranslationally switch onto the tmRNA ORF, and the existence and function of accessory factors. The work in several of these areas is still ongoing, but important progress has been made nonetheless. One study designed to identify bacterial proteins that copurify with the SmpB•tmRNA complex obtained the exoribonuclease RNase R (94). A follow-up study has determined that RNase R functions in the degradation of *trans*-translation-activating mRNAs that are truncated or contain rare codons (150). The ribonucleolytic function associated with *trans*-translation serves to prevent subsequent and continued ribosome stalling events on such templates (125, 150, 201), and is emphasized in eukaryotic mRNA surveillance pathways (39, 48). Notably, RNase R has also been shown to directly regulate the cellular levels of tmRNA in the bacterium *Caulobacter crescentus* (75). Further investigations should help explain how the activities of RNase R are efficiently integrated into the *trans*-translation pathway.

Biochemical studies of the nucleotides just upstream of the tmRNA MLD have offered glimpses into the regulation of tmRNA tag translation. Since the tag-encoding ORF is an inherent feature of tmRNA and is connected to the tmRNA TLD, ribosome engagement on tmRNA does not require the mRNA-ribosome association events that define translation initiation. Therefore, the absence of a Shine-Dalgarno sequence upstream of the ORF may be easily rationalized, though it is already known that standard mRNAs lacking upstream ribosome binding sequences are present and translationally functional in bacteria (9, 87,

126). More distinctive, then, is that the tmRNA ORF does not begin with a start codon. Therefore, it is presumed that the mechanism by which the ribosome resumes translation on the tmRNA ORF is unique and might require ancillary components. Certain nucleotides upstream of the tmRNA translation resume codon are highly conserved and the results of recent studies have shown that the modification of these nucleotides produces null or partially defective tmRNA variants (108, 113, 190). It has also been proposed that SmpB contacts the region immediately upstream of the tmRNA ORF and, thus, helps determine the ORF resume codon (108).

Additional Cellular Factors Associated with *trans*-Translation

It became apparent early on that several cellular components that function in translation are also important for tmRNA and SmpB function in *trans*-translation. Alanyl-tRNA synthetase catalyses the addition of an alanine charge onto tmRNA (107, 182), EF-Tu is required for the efficient delivery of tmRNA and SmpB to the ribosome in a quaternary SmpB•tmRNA•EF-Tu•GTP complex (11, 156), and tmRNA and SmpB must associate with the 70S ribosome in order to mediate *trans*-translation (106, 177, 182). These functional associations have been pivotal for our understanding of tmRNA and SmpB function. Therefore, the identification of addition cellular factors that interact with tmRNA and SmpB has been an important effort throughout the history of *trans*-translation research.

To specifically isolate factors that could associate with tmRNA and SmpB protein within a ribonucleoprotein complex, His_6-SmpB and tmRNA were expressed in *E. coli* and affinity-purified using Ni^{2+}-nitrilotriacetic acid (Ni^{2+}-NTA) chromatography (94). The [His_6-SmpB]•tmRNA complex (hereafter referred to as the SmpB•tmRNA complex) was further purified using size exclusion chromatography, and its protein components were resolved using SDS-PAGE. The most represented copurified proteins were characterized using Edman degradation and matrix-assisted laser desorption ionization/time-of-flight (MALDI/TOF) mass spectrometry. This investigation led to the identification of four proteins associated with the SmpB•tmRNA complex: ribosomal protein S1, ribonuclease R (RNase R; also known as VacB), phosphoribosyl pyrophosphate synthetase (PrsA), and an unknown protein that is encoded by the gene *yfbG* (94).

The isolation of ribosomal protein S1 from the SmpB•tmRNA complex complemented and confirmed S1•tmRNA binding studies conducted by other groups (151, 197). Ribosomal protein S1 is a large, essential protein (105) that binds weakly and reversibly to the small 30S subunit (170). S1 consists of a string of six S1 RNA binding domains and is believed to function in translation through protein-RNA interactions and protein-protein interactions (170). In particular, ribosomal protein S1 makes contacts with mRNAs during translation initiation (19). These contacts are especially important for the translation of mRNAs lacking Shine-Dalgarno sequences (151, 170). tmRNA does not have a Shine-Dalgarno sequence upstream of its tag ORF, and, thus, S1 had been

suggested to facilitate the resumption of translation on the mRNA-like domain of tmRNA during *trans*-translation (94). The conformational change observed in tmRNA that results from S1 association has also been implicated in promoting translation of the tmRNA reading frame, possibly by relieving strain in or exposing that region (21, 197). However, the results of studies showing that *trans*-translation is functional in the absence of ribosomal protein S1 has challenged the relevance of S1•tmRNA interaction, especially on the ribosome as opposed to in solution (123, 146, 157). Further experiments designed to specifically study the functional association of S1 and tmRNA should help clarify the physiological significance of their interaction.

The 3'-to-5' exoribonuclease RNase R, one of fifteen ribonucleases known to be present in *E. coli* (37, 195), is encoded by the *rnr* gene and was named based on its preference for degrading highly structured RNA such as ribosomal RNA (31, 32). RNase R contains an N-terminal cold shock domain and a C-terminal S1 domain and possesses the distinctive capacity to independently degrade highly structured RNA substrates that are otherwise refractory to degradation by the other exoribonucleases polynucleotide phosphorylase (PNPase) and RNase II (184). Obvious explanations for the copurification of RNase R with the SmpB•tmRNA complex include possible roles for RNase R in tmRNA processing and turnover and in the degradation of mRNAs released from stalled ribosomes. One study showed that *rnr* is induced under the *E. coli* cold shock response, resulting in a larger cellular pool of RNase R that is important for promoting the maturation of tmRNA under cold shock (25). Another study found

that RNase R functions in the cellular turnover of tmRNA in *C. crescentus* (75). The SmpB•tmRNA copurification study determined that tmRNA-mediated tagging is elevated and exhibits a distinctive pattern in *rnr* mutant cells compared to wild-type cells (94). This finding is consistent with observations that RNase R functions in the degradation of aberrant, *trans*-translation-activating mRNAs that lack in-frame stop codons or contain rare codons (150). RNase R will likely be the subject of further scrutiny based on its many functional implications in *trans*-translation.

PrsA plays an integral role in the *de novo* synthesis of nucleotides, tryptophan, and histidine (77, 78). Electrophoretic mobility shift assays suggested that PrsA and tmRNA associate with low specificity, and that a more specific interaction might require other factors or base modifications of tmRNA (94). Additionally, *prsA* mutant cells exhibit normal tmRNA-mediated protein tagging (94). A link between *prsA* and tmRNA had been established previously, when a screen for temperature-sensitive mutants of an *E. coli ssrA* mutant produced two *prsA* mutants whose temperature-sensitive phenotypes could be rescued by wild-type *ssrA* (5). However, a general association between tmRNA function and the suppression of conditional phenotypes has also been observed (131). A clear, defined link between tmRNA and SmpB function and PrsA remains to be established.

Chapter 2: A Transposon Mutagenesis-Based Screen for Genes that Influence *trans*-Translation

Summary

A genetic screen was carried out to identify genes that are important for *trans*-translation in *E. coli*. A Tn5-based transposon was used to generate *E. coli* transposon insertion mutants and bacteriophage phenotyping assays were used for mutant screening. The transposon contained two key features: a kanamycin resistance gene that allowed for the selection of transposon insertion mutants and a conditional origin of replication that was used for transposon rescue. The two λ-P22 hybrid bacteriophages λ*imm*P22 *c2-5 dis* and λ*imm*P22 *c1-7 dis* were used to screen transposon insertion clones. Phage λ*imm*P22 *c2-5 dis* is dependent on *trans*-translation and is unable to lyse *E. coli ssrA* and *smpB* mutants, while phage λ*imm*P22 *c1-7 dis* is able to lyse wild-type cells and *ssrA* and *smpB* mutants indiscrimately. Three independent transformations of *E. coli* with the transposon generated 20,673 mutants, 18,949 of which were assayed for resistance to bacteriophage λ*imm*P22 *c2-5 dis*, producing 148 primary candidates with phage phenotypes similar to *E. coli ssrA* and *smpB* mutants. The 148 primary candidates were screened for sensitivity to phage λ*imm*P22 *c1-7 dis* to remove clones that were generally resistant to phage infection, leaving 16

secondary candidates. The transposon insertion sites in the secondary candidates were mapped through transposon rescue and outward sequencing from the ends of the transposon. *ssrA* and *smpB* were each identified once in this screen. The remaining candidate genes were known to function in proteolysis or at the cell envelope. While most of the secondary candidates appeared to function normally in an endogenous protein tagging assay designed to indicate *trans*-translation activity, mutants that carried transposon insertions in *lon* were observed to have abnormally high levels of tagged proteins.

Introduction

Our knowledge of *trans*-translation had opened with the discovery of tmRNA and the investigation of the peptide tag encoded by tmRNA. The identification of cellular factors that support tmRNA function has greatly assisted our understanding of the mechanism of *trans*-translation and underscores the significance of studying the physical associations of biological molecules. However, most of these functional linkages involved proteins associated with translation and extensive searches had not been performed for genes that are specifically important for *trans*-translation. With the objective of further characterizing the physiological significance of *trans*-translation, I considered possible methods for identifying novel genes that are functionally linked to the pathway.

One method I designed involved the screening of *E. coli* mutants generated through transposon mutagenesis. This screen involved the use of a Tn5-based transposon with two key features. A kanamycin resistance gene supported the selection for *E. coli* mutants carrying integrated transposon using growth medium containing kanamycin. A conditional origin of replication allowed for transposon rescue and integration site mapping. To identify relevant mutants, I considered the various phenotypes associated with bacterial *ssrA* and *smpB* mutants, which are diverse and sometimes subtle. They include the inability to support the development of certain temperate bacteriophages (95, 148, 191), temperature sensitivity and reduced motility (95, 107, 137), the loss of pathogenicity (14, 88, 92, 137), diminished survival in macrophages (13, 137), and inviability (80, 81, 96, 187). For the *E. coli* mutants I generated in my screen, I decided that the best phenotype to use for initial screening was the inability to support the growth of hybrid bacteriophage λ*imm*P22 *c2-5 dis*.

The lambdoid bacteriophages are grouped together based on similar genome organization and gene regulation (26, 27). Included within this group are the famous *E. coli* phage λ and the less well-known *Salmonella* Typhimurium phage P22. *E. coli* surface proteins are not recognized as attachment sites for phage P22 and, thus, *E. coli* cells are not susceptible to phage P22 infection. However, the P22 genome is organized very similarly to the λ genome and contains several genes with functions that are analogous to those present in λ. It follows that certain hybrid phages resulting from recombinant crosses between P22 and λ are able to infect and lyse *E. coli* (22, 55, 200). Specifically, these

hybrid phages retain the immunity, or regulatory, functions and replication genes of P22 but express the morphogenic genes of λ.

An interesting feature of P22 is that it has two immunity regions while λ has one. The P22 immunity region *imm*C is analogous to the λ immunity region and encodes the C2 repressor and key regulatory proteins C1 and C3, which are analogous to λ proteins CI, CII, and CIII, respectively. The P22 immunity region *immI*, which has no homolog in λ, encodes additional regulatory proteins, including the antirepressor protein Ant. The two *E. coli*-infective λ*imm*P22 hybrid phages used in this study contain both immunity regions, the recombination and integration genes, and the replication genes of P22. *E. coli ssrA* and *smpB* mutants do not support the lytic development of such phages, a phenotype that is dependent on the proper functioning of the P22 *c1* gene (149, 169). λ*imm*P22 *c2-5 dis*, which has functional *c1*, exhibits severely inhibited growth in *ssrA* and *smpB* mutants. The *c1*-defective λ*imm*P22 *c1-7 dis* appears to grow normally in *ssrA* and *smpB* mutants. It is possible that the regulatory capacity of the P22 C1 protein is dependent on normal *trans*-translation function and that abnormal C1 activity in *ssrA* and *smpB* mutants leads to the inability of the phage to transition from lysogenic phase into lytic development. The ability of *trans*-translation to modulate the levels of a regulatory protein has been shown with the *E. coli lac* operon (1).

Materials and Methods

Bacterial strains, bacteriophages, and plasmids. Bacterial strains were cultivated in LB medium (10 g tryptone, 5 g yeast extract, and 5 g NaCl per liter). Antibiotics (ampicillin [100 μg/uL], kanamycin [50 μg/mL], or tetracycline [24 μg/mL]) were added to the bacterial medium when appropriate. *Escherichia coli* K-12 derivative W3110 [F^- λ^- IN(*rrnD-rrnE*)1 *rph-1*] was utilized as wild-type and is the parental strain of the mutants analyzed in this study. Strains *ssrA*::kan (107) and Δ*smpB-1* (95) have been described previously. Strains *clpA*::kan and *clpP*::cm were prepared through phage P1 transduction of W3110 with P1 lysates of SG22176 and SG22174 (60), respectively. Strain *clpX*::kan was prepared through P1 transduction of W3110 with P1 lysate of BW25113 *ΔclpX* from the Keio collection (8). The λ-P22 hybrid bacteriophages λ*imm*P22 *c2-5 dis* and λ*imm*P22 *c1-7 dis* have been described previously (169). Plasmid pKW11 is derived from pKW1 and has a p15A origin of replication, confers resistance to tetracycline, and expresses *E. coli* wild-type *ssrA* under the control of the native *ssrA* promoter (95, 153). Plasmid pKW24 is a derivative of pKW11 that encodes variant $tmRNA^{H6}$ (152). The $tmRNA^{H6}$ variant encodes a modified degradation tag (NH_2-[A]ANDEHHHHHH-COOH) that is poorly recognized by proteases, in contrast to the wild-type tag (NH_2-[A]ANDENYALAA-COOH).

Transposon mutagenesis of *E. coli* W3110. Transposon EZ::TN <R6Kγ*ori*/KAN-2> (Epicentre Biotechnologies) was used to generate transposon insertion mutants of *E. coli* W3110. This transposon has two key features

facilitating its use in my screen. The first is a kanamycin resistance gene, Tn903 kan^R, that serves as a selectable marker for cells that carry integrated transposon, and the second is a conditional origin of replication that allows transposon rescue cloning and mutant identification. Electrocompetent *E. coli* W3110, generated as described (158), was electroporated (2.0 kV, 200 Ω, 25 μF) with transposon complexed with transposase (57). Insertion clones were selected for on LB containing kanamycin. Kanamycin-resistant colonies were screened based on bacteriophage growth phenotype using bacteriophage cross-streak assays.

Bacteriophage cross-streak assay. The amount of phage to use for the bacteriophage cross-streak assay was determined by titration for a dilution of phage stock that would cause severe lysis of wild-type but no lysis of *ssrA* and *smpB* mutants. To perform the cross-streak assay, phage was applied onto the midline of an LB plate marked with assay lanes. Insertion clones were streaked across the phage spot in each lane. Assay plates were then incubated at 37°C. Bacterial growth during each assay was recorded twice, after approximately seven and twenty-two hours of incubation. Sensitive clones showed a clearing of growth at the phage region of the cross-streak. Clones assayed with λ*imm*P22 *c2-5 dis* that showed no lysis or less lysis than that observed for wild-type were collected as primary candidates. To screen out clones that were resistant to phage infection as a result of mutations that affected processes independent of *trans*-translation, primary candidates were tested for sensitivity to λ*imm*P22 *c1-7 dis*, which grows in wild-type, *ssrA* mutant, and *smpB* mutant cells

indiscriminately. Primary clones that were sensitive to lysis by λ*imm*P22 *c1-7 dis* were retained as secondary candidates.

Colony PCR analysis. Colony PCR was performed with all of the screen primary candidates to assess the nature of their *smpB* and *ssrA* genes. In the genome of *E. coli* and several other bacterial species, *smpB* and *ssrA* are immediately adjacent to each other. In addition to supporting a functional association for *smpB* and *ssrA*, this organization also allows easy PCR analysis of the two genes. Initial PCR analysis was carried out with primers 5'-PROsmpBssrA (5'-CGGGTTCATGCTAAGATAGAG-3') and PROsmpBssrA-3' (5'-GGACTTCATCGGATGACTCTG-3'), which were designed to amplify a region encompassing *smpB* and *ssrA*. Complete high magnesium concentration PCR reactions contained a small smear (carried on the end of a 20 μL plastic micropipette tip) of a single colony, 1X ThermoPol reaction buffer containing $MgSO_4$ (New England Biolabs), 2.5 mM of additional $MgSO_4$, 0.2 mM of dNTPs (Amersham Biosciences), 0.5 μM of each primer, and 2.5 U of Vent DNA polymerase (New England Biolabs) in a 50 μL total volume. The PCR cycling parameters were as follows: 95°C for 5 m, 5 cycles of (95°C for 30 s, 51°C for 30 s, and 72°C for 3.5 m), 25 cycles of (95°C for 30 s, 58°C for 30 s, and 72°C for 3.5 m), 72°C for 10 m, and finish hold at 4°C. PCR products were analyzed based on electrophoretic mobility through agarose gel (1%). Candidates determined to have larger than expected PCR products in the *smpB-ssrA* genomic region were further analyzed using PCR primers designed to amplify an *smpB*-specific or *ssrA*-specific region. The primers used to study *smpB* were 5'-

PROsmpBssrA, 5'-smpB (5'-CCGATGATTCACGACGCTTATG-3'), and smpB-3' (5'- CTGGTCAATAATTGGAGTGCAG-3'). The primers used to study *ssrA* were 5'-ssrA (5'-CCGACACAAATGTTGCCATC-3') and PROsmpBssrA-3'. The PCR cycling parameters that were used for the separate analyses of *smpB* and *ssrA* were the same as those described above except for a decrease in extension time from 3.5 m to 2.7 m.

Transposon rescue and mapping. Transposons were rescue cloned and used in DNA sequencing reactions to map their integration sites in the secondary candidates. Genomic DNA was purified from the secondary candidates using the QIAamp DNA Mini Kit (Qiagen) and digested using the restriction endonuclease BsaHI (New England Biolabs). Purified restriction fragments were treated with T4 DNA Ligase (New England Biolabs). Purified DNA from ligation reactions were electroporated into TransforMax EC100D *pir*+ *E. coli* (Epicentre Biotechnologies). This strain of *E. coli* expresses the π protein, encoded by the *pir* gene, which is required for the replication of vectors containing the R6Kγ origin of replication. Therefore, circularized genomic DNA fragments that contain the EZ::TN <R6Kγ*ori*/KAN-2> transposon are propagated as plasmids in TransforMax EC100D *pir*+ *E. coli*. *E. coli pir*+ transformants carrying rescue cloned transposon were selected for using LB agar containing kanamycin. Purified plasmids were used as templates in DNA sequencing reactions (Genewiz) prepared with an outward transposon primer, KAN-2 FP-1 or R6KAN-2 RP-1 (Epicentre Biotechnologies), to determine the integration site of the transposon in each clone.

Endogenous tagging assay. Strains carrying pKW510 or pKW540 were cultivated in LB containing ampicillin at 37°C and 200 rpm, until the culture OD_{600} was ≈ 1.0. Cell harvests were normalized to 50 mL of culture with an OD_{600} of 1.0. Pelleted cells were resuspended in freshly prepared urea lysis buffer (8 M urea, 1% Triton X-100, 2 mM β-mercaptoethanol, 100 mM NaH_2PO_4, and 10 mM Tris-HCl [pH 8.0]). Following ten minutes of mixing at room temperature, cells were mechanically disrupted by sonication. His_6-tagged proteins in clarified lysates were purified using Ni^{2+}-NTA chromatography. Bound proteins were eluted from Ni^{2+}-NTA resin using freshly prepared elution buffer (8 M urea, 1% Triton X-100, 10 mM β-mercaptoethanol, and 0.1 M acetic acid). Purified proteins were analyzed by SDS-PAGE (7) and Western blotting with a mouse monoclonal anti-His_6-horseradish peroxidase (HRP) probe (Santa Cruz Biotechnology).

Results

Transposon insertion mutant generation and primary screening. The dependence of λ*imm*P22 *c2-5 dis* on *ssrA* and *smpB* function for lytic development (95, 149, 191) was incorporated into the design of a genetic screen for genes that are important for *trans*-translation (Fig. 2.1). Transposon EZ::TN <R6Kγ*ori*/KAN-2> was used to generate a large number of *E. coli* mutants for screening. The transposon was introduced into *E. coli* wild-type strain W3110 via electroporation and LB medium containing kanamycin was used to select for

transposon insertion clones. I performed three transformations of W3110 with the transposon and, thus, generated 20,673 insertion clones.

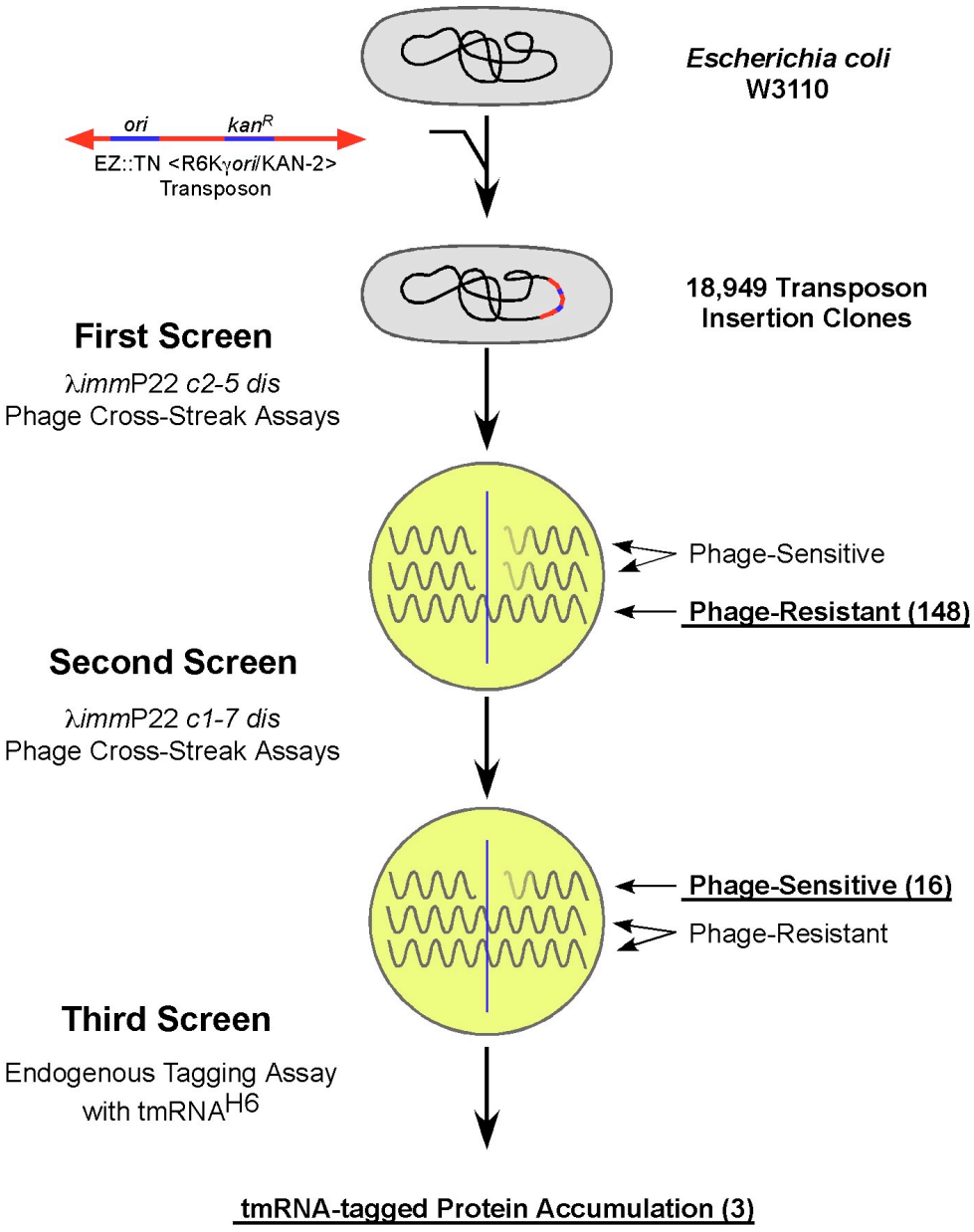

Fig. 2.1. A transposon mutagenesis-based screen for genes that function in *trans*-translation. A transposon was introduced into *E. coli* wild-type strain W3110 to generate transposon insertion clones. These clones were screened for resistance to hybrid phage λ*imm*P22 *c2-5 dis* (unable to grow on *ssrA* and *smpB* mutants) to isolate primary candidates. The primary candidates were screened for sensitivity to hybrid phage λ*imm*P22 *c1-7 dis* (able to grow on *ssrA* and *smpB* mutants) to identify secondary candidates. The secondary candidates were assessed for *trans*-translation function using an endogenous protein tagging assay, which determines the ability of a strain to produce tmRNA-tagged proteins. Three secondary candidates were observed to accumulate abnormally high levels of tmRNA-tagged proteins.

Using phage cross-streak assays, 18,949 insertion clones were screened for the inability to support the growth of λ*imm*P22 *c2-5 dis*. 148 insertion clones (primary candidates) were observed to exhibit resistance to λ*imm*P22 *c2-5 dis* relative to wild-type cells. However, many of these clones were not as resistant to the phage as *ssrA* and *smpB* mutants. This suggested that *ssrA* and *smpB* play unique roles during the lytic development of λ*imm*P22 *c2-5 dis*. In addition to not supporting optimal growth of λ*imm*P22 *c2-5 dis*, the primary screen candidates could be distinguished based on a few common characteristics. 83 primary candidates grew slower than wild-type cells, 10 of which were particularly slow-growing, 85 exhibited mucoidy, and 3 exhibited a drier morphology than wild-type cells.

Colony PCR analyses. In the *E. coli* chromosome, the *smpB* and *ssrA* genes are located immediately adjacent to each other. This organization allowed for the relatively simple and simultaneous PCR analysis of both genes (Fig. 2.2A). Initial colony PCR analysis was performed with the primary candidates using a pair of primers that were designed to amplify a 1.2 kb region of genomic DNA containing *smpB* and *ssrA*. Insertion of the 2 kb transposon into *smpB* or *ssrA* was expected to be easily determined, due to a size shift of the PCR product. Therefore, clones with intact *smpB* and *ssrA* were expected to produce 1.2 kb PCR products, while clones carrying transposon in *smpB* or *ssrA* were expected to produce 3.2 kb PCR products. The results of at least three independent PCR reactions with each candidate showed that only candidates A31 and B65 produced 3.2 kb PCR products instead of 1.2 kb PCR products

(Fig. 2.2B). My interpretation of these results was based on the presence or absence of the 1.2 kb PCR product. While several clones produced higher molecular weight PCR products, they mainly produced 1.2 kb products. On the other hand, the two clones that produced 3.2 kb PCR products did not produce the 1.2 kb species that would be amplified from the *smpB* and *ssrA* target region lacking a transposon. Further analyses of candidates A31 and B65 were performed using PCR primers designed to specifically amplify *smpB* or *ssrA* only (Fig. 2.2A and data not shown), confirming that A31 contained a transposon in *ssrA* and B65 contained a transposon in *smpB*.

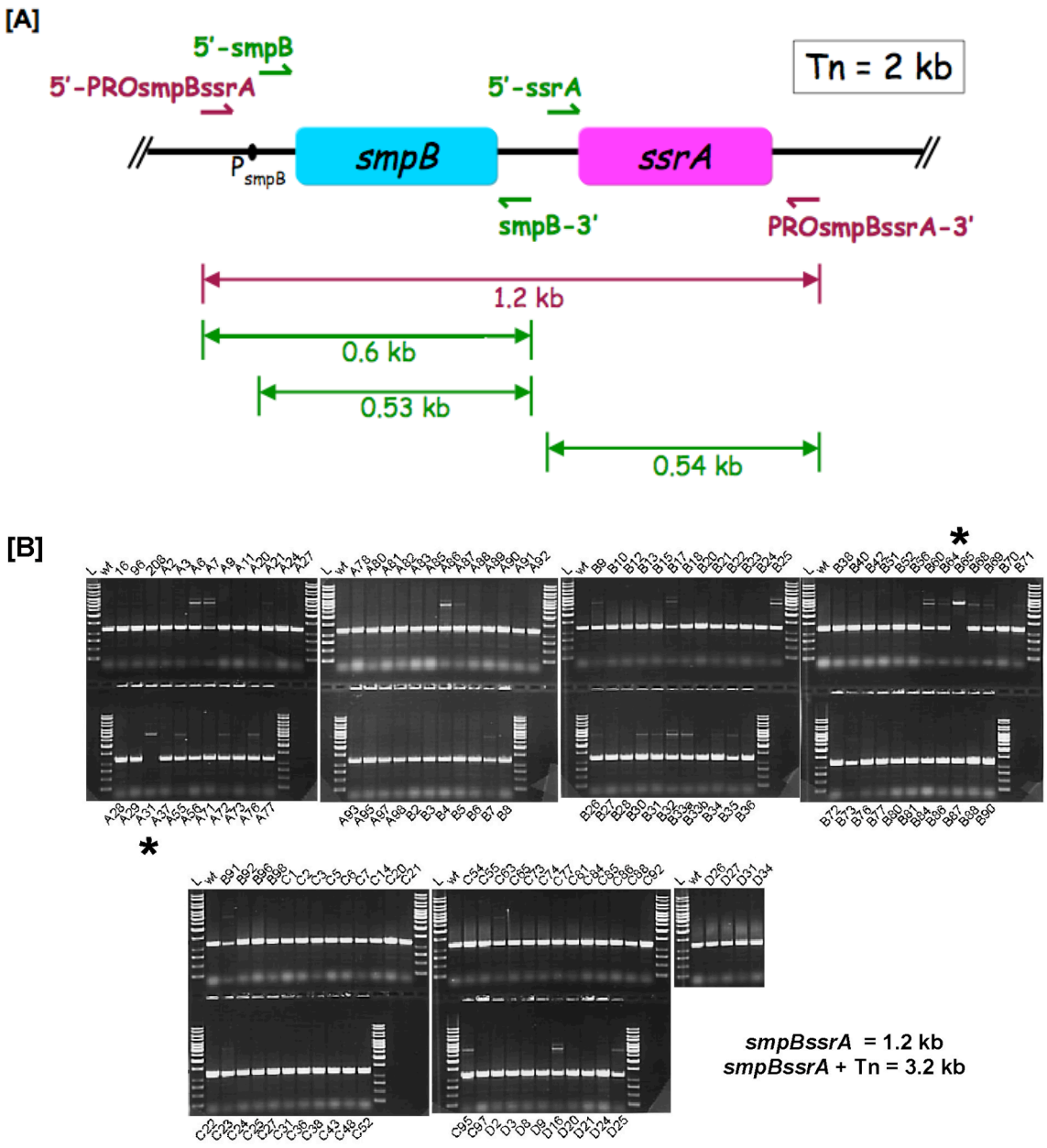

Fig. 2.2. Colony PCR analyses of mutagenesis screen primary candidates. [A] Various primers were designed for PCR analyses of the *smpB* and *ssrA* genes in the screen candidates. The transposon size was 2 kb and PCR extension times were determined accordingly to permit amplification of target regions containing transposon. P_{smpB}, *smpB* promoter. [B] Colony PCR analysis using primers 5'-PROsmpBssrA and PROsmpBssrA-3' showed that candidates A31 and B65 produced 3.2 kb PCR products (asterisks), suggesting transposon insertions in the *smpB* and *ssrA* region. Reactions were analyzed by agarose gel (1%) electrophoresis. Screen candidates were given arbitrary names prior to transposon mapping; Tn, transposon; L, DNA ladder; wt, wild-type.

Mutant secondary screening. Hybrid phage λ*imm*P22 *c1-7 dis*, which does not depend on SmpB and tmRNA function for growth, was used to screen out primary candidates that were generally resistant to phage infection rather than abnormal in some aspect of *trans*-translation (Fig. 2.1). Based on this distinction, insertion clones that were resistant to both λ*imm*P22 *c2-5 dis* and λ*imm*P22 *c1-7 dis* were considered likely to contain mutations in genes that prevent phage infection and/or growth rather than in genes that might have a *trans*-translation related function. Phage cross-streak assays using hybrid phage λ*imm*P22 *c1-7 dis* narrowed the 148 primary candidates to 16 secondary candidates (Fig. 2.1).

Transposon integration site mapping. Transposons from the 16 secondary candidates were rescue cloned in order to map their integration sites. Table 2.1 lists the transposon integration sites in the secondary candidates. Through my integration site mapping, I confirmed the insertions in *ssrA* and *smpB* that were suggested by my colony PCR results. My identification of *ssrA* and *smpB* was encouraging and validated my approach for identifying genes that function in *trans*-translation. Interestingly, among the remaining secondary candidates were three with independent insertions in the *lon* gene, hereafter designated *lon-1*, *lon-2*, and *lon-3* (Fig. 2.3). The *lon* gene encodes the ATP-dependent protease Lon, which contains three distinct domains: an amino-terminal domain of undefined function, a central ATPase domain crucial for substrate binding and unfolding, and a C-terminal peptidase domain. Since substrate binding, unfolding, and proteolysis are coupled functional events, it was

expected that the mutation of either the ATPase domain or the peptidase domain of Lon should render the protein nonfunctional in the cell (2, 51, 167). DNA sequencing analysis of the *lon*::Tn mutants revealed transposon disruptions in the region encoding the ATPase domain in mutants *lon-1* and *lon-2*, and in the region encoding the peptidase domain in *lon-3* (Fig. 2.3).

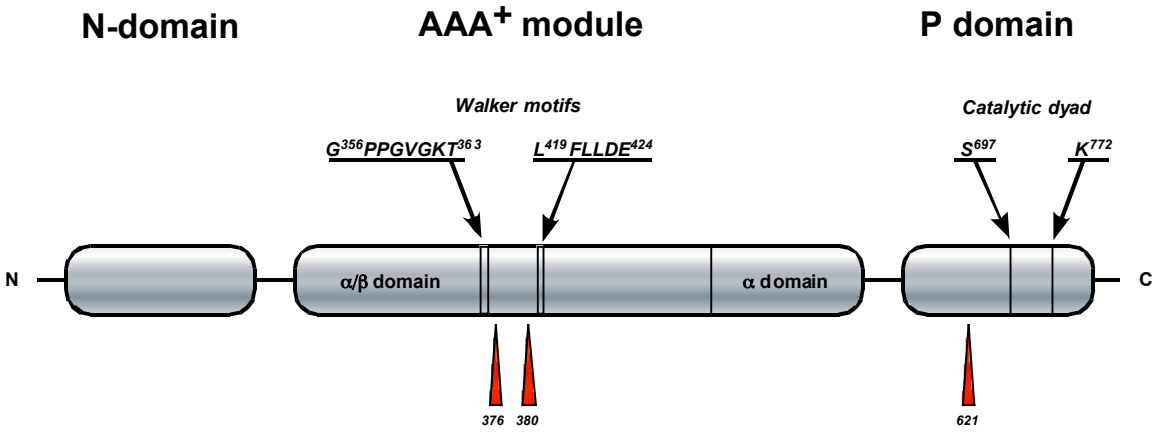

Fig. 2.3. Transposon integration sites in *lon*::Tn mutants. A schematic representation of Lon protease domains is shown. The function of the Lon N-terminal domain is undefined. The AAA^+ module, or ATPase domain, is crucial for substrate recognition and unfolding by Lon. The P domain confers proteolytic function to Lon. Red arrowheads indicate transposon integration sites in *lon-1* (residue 376), *lon-2* (residue 380), and *lon-3* (residue 621).

Gene Disrupted	# of Hits	Functional Description
ssrA	1	*trans*-translation factor
smpB	1	*trans*-translation factor
lon	3	ATP-dependent protease; degradation of abnormal cellular proteins
hflK	1	Part of modulator for ATP-dependent protease FtsH
tolQ	1	Cell envelope integrity; uptake of group A colicins and filamentous phage DNA
tolR	1	Cell envelope integrity; uptake of group A colicins and filamentous phage DNA
tolA	1	Cell envelope integrity; uptake of group A colicins and filamentous phage DNA
mdoH	1	Membrane glycosyltransferase; cell envelope biogenesis
rfaH	1	Transcription antiterminator; expression of outer membrane and secreted proteins
surA	4	Peptidyl-prolyl cis-trans isomerase; outer membrane protein maturation
yciS	1	Hypothetical inner membrane protein; function unknown

Table 2.1. Transposon integration sites in transposon mutagenesis screen secondary candidates. The transposons in the screen mutants were rescued with flanking host sequences and used as templates for DNA sequencing reactions containing outward primers that were complementary to the ends of the transposon.

Aside from *ssrA*, *smpB*, and *lon*, the genes identified in this screen appeared to all function at the cell envelope. *hflK* encodes a protein that helps modulate the activity of the inner membrane-associated FtsH protease (99, 100, 103). Since FtsH activity had been observed against tmRNA-tagged reporter proteins (71, 72), I also considered my *hflK* mutant as a potentially promising candidate. Of the remaining secondary candidates, four contained transposon in *surA*, which is a gene that encodes peptidyl-prolyl cis-trans isomerase and functions in outer membrane protein maturation (110). The *tolQRA* operon was disrupted by the transposon three times, once in each of the three genes in the operon. The Tol proteins are known to be generally important for outer membrane integrity (111) and are targeted for the uptake of group A colicins (188). The tenfold reduction of candidates during my secondary phage screening and the emphasis on cell envelope functions represented by my secondary candidate genes also suggested that my primary screening had isolated a significant number of mutants with defects that prevent efficient λ*imm*P22 *c2-5 dis* infection.

Since *lon* was identified multiple times in my screen, I wondered why the genes for the other major cytoplasmic proteases, especially the Clp proteases, were not identified. Phage cross-streak assays performed with *clpA*, *clpX*, and *clpP* mutants confirmed that these mutants exhibit wild-type level sensitivity to the first screening phage λ*imm*P22 *c2-5 dis* (Fig. 2.4). This finding effectively explained why *clp* genes were not identified in my screen. Nevertheless, the identification of *smpB* and *ssrA* in this screen suggested that perhaps one or

more of the other candidates, such as *lon*, might have a *trans*-translation related function.

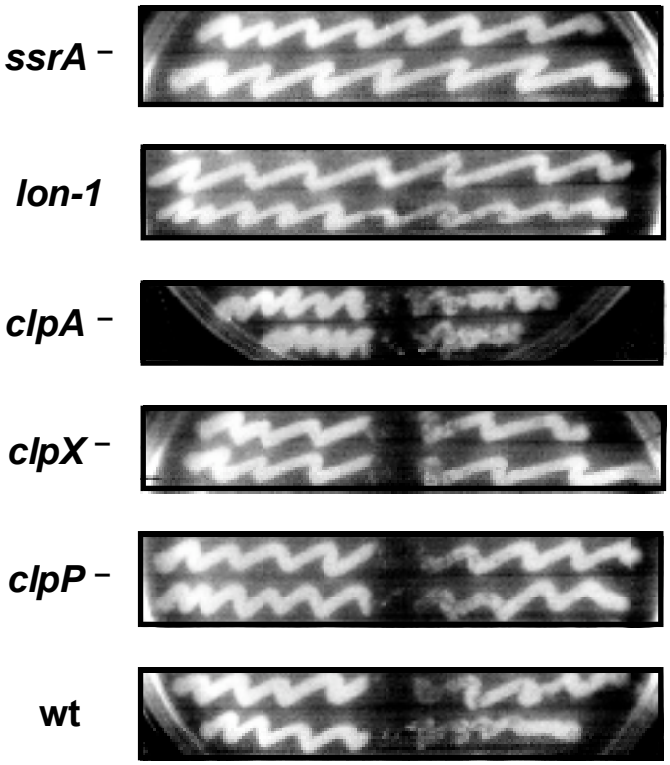

Fig. 2.4. *E. coli clp* mutants are sensitive to lysis by phage λ*imm*P22 *c2-5 dis*. The ability of *clpA*, *clpX*, and *clpP* mutants to support the lytic development of λ*imm*P22 *c2-5 dis* was assessed using the phage cross-streak assay. *ssrA* and *lon* mutant cells were resistant to phage, while *clpA*, *clpX*, and *clpP* mutant cells exhibited wild-type level sensitivity to phage. wt, wild-type.

Assessment of *trans*-translation in screen secondary candidates. To characterize the screen secondary candidates based on *trans*-translation function, I decided to determine whether the selected candidates had a defect in the SmpB-tmRNA tagging process and potential downstream functions. To this end, I utilized an endogenous protein tagging assay mediated by $tmRNA^{H6}$. This assay has been used extensively and has greatly facilitated a better

understanding of the *trans*-translation process (45, 94, 127, 152, 171). The degradation sequence encoded by wild-type tmRNA is known to target tmRNA-tagged proteins for rapid destruction by cellular proteases such as ClpXP, ClpAP, FtsH, and Tsp (60, 72, 98), thus making it difficult to evaluate defects in *trans*-translation or to analyze the nature and identity of tagged proteins. Conversely, proteins modified by $tmRNA^{H6}$, a variant of tmRNA encoding a tag that ends with a His_6 epitope rather than the wild-type tag, are significantly stabilized and can be purified using Ni^{2+}-NTA chromatography and detected by Western blot analysis (45, 94, 152, 171, 196). It should be noted that in a *trans*-translation-competent strain, chromosomally encoded wild-type tmRNA is able to compete with $tmRNA^{H6}$, resulting in an apparent overall reduction of $tmRNA^{H6}$-tagged protein levels. This effect can be observed in comparing the tagging profile of a wild-type strain to the tagging profile of an *ssrA* mutant (Fig. 2.5A, lanes 1 and 3). My use of the endogenous protein tagging assay was thus intended to provide an approximation of mutant cell tagging ability relative to wild-type cells in cases where the mutant was not completely defective in *trans*-translation. To determine the extent of *trans*-translation function in the secondary candidates, their levels of $tmRNA^{H6}$-tagged proteins were compared to those observed in the otherwise isogenic parental wild-type strain.

The wild-type strain carrying the $tmRNA^{H6}$ variant produced a characteristic set of $tmRNA^{H6}$-tagged proteins (Fig. 2.5A, lane 1). His_6-tagged proteins were not observed when the *ssrA*::Tn clone was complemented with wild-type tmRNA (Fig. 2.5A, lane 2), confirming that the tagged proteins observed

in the wild-type strain were produced via tmRNA^{H6} activity. As expected, complementation of clone *ssrA*::Tn with tmRNA^{H6} resulted in the accumulation of higher levels of tagged proteins compared to wild-type due to the absence of tmRNA competition (Fig. 2.5A , lanes 1 and 3). Clone *smpB*::Tn did not produce tmRNA^{H6}-tagged proteins, as tmRNA function cannot compensate for the strict requirement of SmpB activity in *trans*-translation (Fig. 2.5A, lane 4).

All of the remaining secondary candidates produced tmRNA^{H6}-tagged proteins, indicating that they were all functional in the protein tagging capacity of *trans*-translation (Fig. 2.5B). However, the *lon*::Tn (*lon-1*) mutant accumulated higher levels of tmRNA^{H6}-tagged proteins than wild-type cells (Fig. 2.5A, lane 1 and Fig. 2.5B, lane 5). Similarly, I observed an increased accumulation of tmRNA^{H6}-tagged proteins in the other two *lon*::Tn mutants *lon-2* and *lon-3* (Fig. 2.5C). The results of my endogenous tagging assays emphasized *lon* as a strong candidate for *trans*-translation related function.

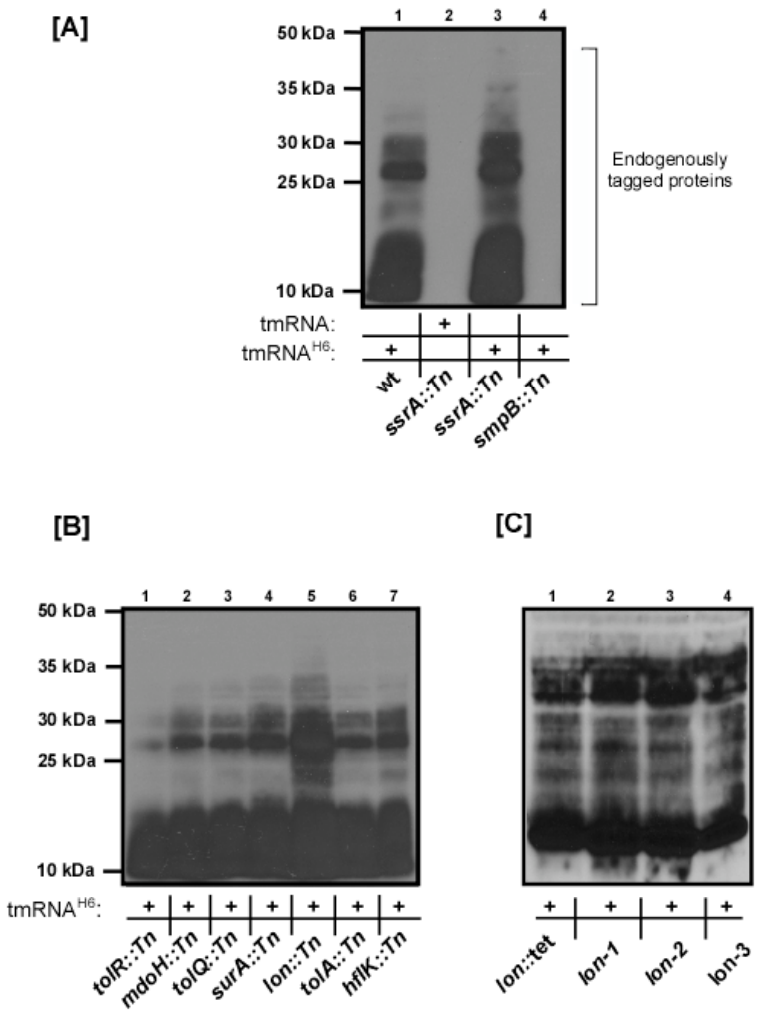

Fig. 2.5. Endogenous protein tagging assays performed with selected secondary candidates and wild-type tmRNA or variant tmRNA^{H6}. Cells expressing plasmid-borne wild-type tmRNA or variant tmRNA^{H6} (indicated by plus signs) were grown in liquid culture until an OD_{600} of ≈ 1.0. Purified His_6-tagged proteins were analyzed by SDS-PAGE and detected by Western blotting using HRP-conjugated anti-His_6 antibody. [A] Mutant *ssrA*::Tn had higher levels of tmRNA^{H6}-tagged proteins than wild-type cells due to the absence of functional wild-type tmRNA (lanes 1 and 3). Wild-type tmRNA-tagged proteins were not detected in this assay (lane 2). Mutant *smpB*::Tn was completely defective in the tagging function of *trans*-translation (lane 4). [B] Compared to wild-type cells, the *tolR*::Tn, *tolQ*::Tn, and *tolA*::Tn mutants showed slightly decreased levels of tmRNA^{H6}-tagged proteins (lanes 1, 3, and 6), while the *lon*::Tn (*lon-1*) mutant accumulated higher levels of tmRNA^{H6}-tagged proteins (lane 5). Data shown in panels [A] and [B] of this figure are from different sections of the same exposure of the same blot. [C] Similar endogenous tagging profiles were obtained for *lon-1*, *lon-2*, and *lon-3* and an otherwise isogenic, previously characterized *lon*::tet mutant (from strain AP401). wt, wild-type.

Discussion

By using the distinct bacteriophage phenotype of *E. coli ssrA* and *smpB* mutants to screen for genes that are important for *trans*-translation, I have confirmed the critical importance of *ssrA* and *smpB* and have identified other genes that might influence this mechanism. Aside from *ssrA* and *smpB*, these genes encode proteins that appear to function at the cell envelope or in proteolysis. Despite being identified in my screen, however, the particular mutations caused by transposition into these novel genes did not result in the complete loss of *trans*-translation function when the clones harboring these mutations were tested in my endogenous protein tagging assays. Most of the secondary candidates produced tmRNA^{H6}-tagged proteins at levels comparable to that observed in wild-type cells. One set of candidates, consisting of *tol* mutants, showed a slight decrease in tagged protein levels compared to wild-type cells. Another set of candidates, comprised of various *lon* mutants, accumulated excessively high levels of tmRNA^{H6}-tagged proteins compared to wild-type cells. Nevertheless, it is possible that the remaining secondary candidates are defective in pathways that are associated with *trans*-translation or in unknown stages of *trans*-translation that could not be detected by my endogenous tagging assays. Perhaps the contribution of the secondary candidate genes to *trans*-translation is also enhanced during the lytic development of λ*imm*P22 *c2-5 dis*.

The isolation of *lon* and *hflK* added an unexpected and interesting variable to my screen for genes that are linked to *trans*-translation. I had not expected to identify protease or protease-associated genes in my screen since an investigation of the role of *trans*-translation in the lytic development of hybrid phage λ*imm*P22 *c2-5 dis* suggested that phage growth requires the ribosome rescue function of *trans*-translation, but is only somewhat compromised if the proteolytic function of *trans*-translation is inefficient (191). That study utilized a tmRNA^{DD} variant (98) encoding a tag containing three aspartic acid mutations, which is too charged to be recognized as a substrate for the ClpAP and ClpXP proteases. My endogenous protein tagging assay utilized a tmRNA^{H6} variant, which encodes a tag that is less resistant to proteolysis than the tag encoded by tmRNA^{DD}, but is still poorly recognized by ClpAP and ClpXP. Therefore, the true purpose of my assay was to show the ability of a strain to generate tmRNA-tagged proteins during *trans*-translation, rather than the strain's ability to degrade them. Intriguingly, I found that my *lon* mutants had an abnormal tmRNA^{H6}-tagged protein profile in my endogenous tagging assays, which suggested a *trans*-translation defect. The endogenous tagging assay results I obtained for my *lon* mutants might then indicate an important difference between Lon and Clp recognition of tmRNA-tagged proteins. On the other hand, my *hflK* mutant looked comparable to wild-type cells in my endogenous protein tagging assays. Specifically assessing the stability of a tmRNA-tagged protein, such as Arc-ssrA (71), might reveal the influence of *hflK* on the degradation of tmRNA-tagged proteins.

The identification of *lon*, but not *clpA*, *clpX*, or *clpP*, in my screen was particularly interesting, since ClpAP and ClpXP had already been shown to participate in the degradation of tmRNA-tagged proteins (60). The results of one study suggested that λ*imm*P22 *c2-5 dis* requires only the ribosome rescue function of *trans*-translation for development (191). This selective dependence on *trans*-translation by λ*imm*P22 *c2-5 dis* hints at a feature of bacteriophages in requiring just as much of the host's biology as is needed to support its own development. My phage cross-streak assays with *lon* and *clp* mutants confirmed that *clp* mutants are not defective in growing λ*imm*P22 *c2-5 dis* (Fig. 2.4). The dependence of λ*imm*P22 *c-5 dis* on *lon* but not *clp* further suggests a difference in substrate targeting between proteases during *trans*-translation. The function of *lon* in *trans*-translation might be emphasized for the proper development of λ*imm*P22 *c2-5 dis*. From my screening efforts described in this chapter, *lon* emerged as the most promising gene candidate for *trans*-translation function.

Chapter 3: Lon Protease Degrades Transfer-Messenger RNA-Tagged Proteins

Summary

The *lon* gene encodes the cytoplasmic ATP-dependent protease Lon and was independently isolated three times in the transposon mutagenesis-based screen described in Ch. 2. A complementation assay performed with plasmid-borne *lon* confirmed that a *lon* defect is directly responsible for the phage λ*imm*P22 *c2-5 dis* growth phenotype exhibited by my three screen-derived *lon* mutants. An endogenous protein tagging assay indicated that a previously characterized and widely used *lon* mutant and my screen-derived *lon* mutants each accumulate excessive levels of tmRNA-tagged proteins, suggesting that *lon* might be involved in the cellular degradation of these proteins. To further investigate the possibility that *lon* functions in the turnover of tmRNA-tagged proteins *in vivo*, I used two reporter protein assays designed to measure protein degradation. Reporter protein GFP-ssrA consists of full-length green fluorescent protein C-terminally fused to the tmRNA degradation tag. GFP-ssrA was consistently observed to be more stable in *lon* mutant cells than in wild-type cells. Reporter protein λ-CI-N is encoded by a nonstop mRNA transcript that activates *trans*-translation. λ-CI-N was observed to be efficiently produced and tagged in *lon* mutant cells but the tmRNA-tagged form was stabilized compared to its levels in wild-type cells. Using *in vitro* experiments containing highly purified Lon

protease, I confirmed my *in vivo* results and demonstrated that Lon protease is directly involved in the degradation of tmRNA-tagged proteins.

Introduction

When the model for *trans*-translation was originally proposed, the pathway was described as having two main functions, namely ribosome rescue and targeted proteolysis (98). The idea of a degradative role for tmRNA function and *trans*-translation originated from the realization that the C-terminal residues of the peptide sequence encoded by the tmRNA MLD is similar to the sequence known to be a determinant for proteolytic degradation by intracellular proteases (97, 142, 162, 163). The energy-independent protease Tsp was the first protease shown to specifically degrade a tmRNA-tagged protein (98). However, the activity of Tsp is limited by its localization to the bacterial periplasm and by its restriction to substrates with low stability (97). Therefore, the means of tmRNA-tagged protein turnover in the cytoplasm still needed to be determined and subsequent studies showed that energy-dependent proteases are important contributors to this process (60, 72).

Energy- or ATP-dependent proteases are only found in the cytoplasm, where ATP is available (58, 59). There is no source of ATP in the periplasm or outside of the cell and, therefore, non-cytoplasmic proteases must be ATP-independent and cannot use an ATPase function for denaturing their substrates prior to degradation. The energy-dependent proteases are known to be generally

important for the degradation of various proteins inside the cell, ranging from abnormal proteins to specific substrates that are often regulatory proteins. The flexible specificity of these proteases therefore associates them with several physiological pathways (59).

The energy-dependent proteases of *E. coli* are classed into four families, named after their representative members: ClpAP/ClpXP, HslUV (ClpYQ), FtsH (HflB), and Lon (58). Distinguished from these necessarily cytoplasmic proteases are the energy-independent proteases, which are more numerous and may function in the cytoplasm or periplasm. The Clp and HslUV proteases are two-component proteases consisting of a chaperone subunit, ClpA, ClpX, or HslU, and a peptidase subunit, ClpP or HslV. The ClpA, ClpX, and HslU chaperones are ATPases that are critical for substrate unfolding and substrate translocation into the ClpP or HslV peptidase. The activities of the chaperone components help determine the substrate pools for ClpAP, ClpXP, and HslUV. Unlike the two-component proteases, FtsH and Lon are homomeric proteases derived from a single polypeptide carrying both the chaperone and peptidase functions.

Early work on the proteolytic function of *trans*-translation had shown that ClpXP, ClpAP, and FtsH degrade tmRNA-tagged proteins in a tag-specific manner (60, 72). However, these proteases do not degrade tmRNA-tagged proteins with equal prowess. ClpAP is quite active against tmRNA-tagged protein constructs *in vitro*, but its contribution in *in vivo* assays is less remarkable (47, 60). ClpXP has persisted as the protease mainly responsible for the degradation of tmRNA-tagged proteins *in vivo*. The inner membrane-bound FtsH has

narrower specificity against tmRNA-tagged proteins than ClpAP and ClpXP, and is active mainly on unstable (71) and locally available substrates (101, 102). To further characterize *lon* function in *trans*-translation, I utilized a collection of *in vivo* protein tagging and stability assays. To more accurately measure the effect of a *lon* mutation, I compared my results with those I obtained for *clpX* and *clpA* mutant cells. I found that cells with functional Clp proteases are unable to efficiently degrade tmRNA-tagged proteins in the absence of Lon and that highly purified Lon preferentially degrades tmRNA-tagged proteins *in vitro*. Taken together, my results strongly indicate that Lon participates in the cellular degradation of tmRNA-tagged proteins.

Materials and Methods

Bacterial strains and plasmids. Unless otherwise noted, bacterial strains were cultivated in LB medium. Antibiotics (ampicillin [100 μg/uL], kanamycin [50 μg/mL], or chloramphenicol [30 μg/mL]) were added to the bacterial medium when appropriate. For the *in vivo* protein stability assays, spectinomycin (100 μg/mL) was used to block translation and bacterial growth and to initiate chase. The bacterial strains that were used for this study are described in Ch. 2. Plasmid pPW500 (98) has a pMB1 origin of replication and expresses the reporter *λ-cI-N-trpAt* nonstop mRNA regulated by a P_{TRC} promoter. Plasmid pKW510 is a derivative of pPW500 that additionally expresses wild-type tmRNA regulated by the native *ssrA* promoter. pKW540 is a derivative of pKW510 encoding variant

tmRNA^{H6} rather than wild-type tmRNA. The tmRNA^{H6} variant encodes a modified degradation tag (NH_2-[A]ANDEHHHHHH-COOH) that is poorly recognized by proteases, in contrast to the wild-type tag (NH_2-[A]ANDENYALAA-COOH). Green fluorescent protein variant *gfp*mut3.1 was PCR-amplified from pJBA27 (3), unmodified or fused with the wild-type *ssrA* tag, and subcloned downstream of the P_{BAD} promoter in pBAD18-Cm (65), generating pBAD-GFP and pBAD-GFP-ssrA, respectively.

Complementation of *lon::Tn* mutants. In *E. coli*, *lon* is a single gene locus with its own transcription promoter and terminator. The region of DNA containing *lon* and its flanking promoter and terminator was PCR-amplified from *E. coli* W3110 using primers 5'-TTACTGAATTCATTCTCGGCGTTGAATG-3' and 5'-AGTATGAATTCCATCTAACTTAGCGAGAC-3'. The PCR product was cloned into the EcoRI restriction site of pBR322 (pMB1 origin, amp^R, tet^R) to generate pLon. pLon was transformed into each of the three *lon*::*Tn* mutants by chemical transformation (35). The resultant *lon*::Tn/pLon transformants were tested for complementation of resistance to λ*imm*P22 *c2-5 dis* using the phage cross-streak assay described in Ch. 2.

***In vivo* GFP-ssrA protein stability assays.** Strains carrying pBAD-GFP or pBAD-GFP-ssrA were cultivated in LB containing chloramphenicol at 37°C, 200 rpm, until the culture OD_{600} was ≈ 0.45. Expression of GFP or GFP-ssrA was induced by the addition of 0.01% arabinose to the cultures. Following one hour of induction, cells were gently harvested and washed once with warmed LB, resuspended in one culture volume of warmed LB containing chloramphenicol

and spectinomycin, and returned to incubation at 37°C and 200 rpm. Time-point samples were obtained from the cultures at 0, 10, 20, 40, 60, 90, and 120 min during the chase. The cell harvest from each time-point sample was normalized to one mL of culture at an OD_{600} of 1.0. Cell pellets were resuspended and lysed in 1X SDS sample buffer (7). Total cellular protein was resolved by SDS-PAGE and analyzed by Western blotting with rabbit polyclonal anti-GFP-HRP (Santa Cruz Biotechnology).

***In vivo* λ-CI-N protein stability assays.** Strains carrying pPW500 were cultivated in LB containing ampicillin at 32°C and 200 rpm until the culture OD_{600} was ≈ 0.45. Expression of *λ-cI-N-trpAt* nonstop mRNA was induced by the addition of 1 mM IPTG to the cultures. Following one-half hour of induction, cells were gently harvested and washed once with warmed LB, resuspended in one culture volume of warmed LB containing ampicillin and spectinomycin, and returned to incubation at 32°C and 200 rpm. Time-point samples were obtained from the cultures at 0, 5, 10, 15, 20, and 30 min during the chase. The cell harvest from each time-point sample was normalized to one mL of culture at an OD_{600} of 1.0. Cell pellets were resuspended and lysed in 1X tricine sample buffer (7). Total cellular protein was resolved by Tris-tricine-PAGE (7) and analyzed by Western blotting with mouse monoclonal anti-FLAG M2 (Scientific Imaging Systems) and goat anti-mouse IgG-HRP (Santa Cruz Biotechnology).

Quantification of reporter protein half-life *in vivo.* To extrapolate half-life information for the reporter proteins studied *in vivo*, Western signals were quantified using a GS-710 imaging densitometer and Quantity One software (Bio-

Rad). The quantified protein levels were log-transformed and least-squares fit to an exponential function to obtain decay rate constants. Reporter half-lives were calculated based on first-order decay: $t_{1/2} = \ln(2)/k$.

Protein purification. C-terminally His_6-tagged Lon was purified from *E. coli* strain CH1019 (a gift from the Sauer lab) carrying Lon expression plasmid pET21b-LonH_6 by successive Ni^{2+}-NTA affinity and Mono Q (Amersham Biosciences) anion exchange chromatography. Lon-H_6 expression was induced using 1 mM IPTG for 2 h at 37°C and 200 rpm. Cell pellets were resuspended and mechanically lysed in lysis buffer (1 M NH_4Cl, 20 mM potassium phosphate [pH 7.4], 1 mM EDTA, 2 mM β-mercaptoethanol, and 20 mM imidazole). Clarified lysate was applied to Ni^{2+}-NTA resin and incubated at 4°C for 1 h with agitation. Lon-H_6 was collected from Ni^{2+}-NTA resin using elution buffer (100 mM KCl, 20 mM potassium phosphate [pH 7.4], 1 mM EDTA, 20 mM β-mercaptoethanol, and 250 mM imidazole). Eluate containing Lon-H_6 was applied onto a Mono Q column in buffer A (50 mM KCl, 2 mM EDTA, 20 mM Tris-HCl [pH 7.6], 2 mM β-mercaptoethanol, and 10% glycerol). Lon-H_6 was eluted using a 0 to 60% linear gradient of buffer B (1 M KCl, 2 mM EDTA, 20 mM Tris-HCl [pH 7.6], 2 mM β-mercaptoethanol, and 10% glycerol). The absence of contaminating proteins was verified by SDS-PAGE followed by Coomassie blue staining. Robust Lon activity was determined against fluorescein isothiocyanate-casein (type I; Sigma) by Dr. James Coleman.

The λ-CI-N-trpAt reporter protein (referred to as λ-CI-N protein in this study) has an internal His_6 epitope that was utilized for purification by Ni^{2+}-NTA

chromatography. Untagged and tmRNA-tagged species of λ-CI-N protein were simultaneously purified from an *E. coli clpP clpX lon* triple mutant expressing pPW500. Cells were grown at 37°C and 250 rpm in Terrific Broth (179) containing ampicillin until the culture OD_{600} was ≈ 0.45. Reporter expression was induced for 3 h with 1 mM IPTG. Harvested cells were resuspended and mechanically lysed in lysis buffer (50 mM NaH_2PO_4 [pH 8.0], 300 mM NaCl, and 10 mM imidazole). Clarified lysate was applied to Ni^{2+}-NTA resin, which was then washed with lysis buffer containing 20 mM imidazole. Both forms of λ-CI-N protein were eluted using elution buffer (50 mM NaH_2PO_4 [pH 8.0], 300 mM NaCl, and 250 mM imidazole). The absence of contaminating proteins was verified by Tris-tricine-PAGE followed by Coomassie blue staining.

GFP and GFP-ssrA were purified as described (199). GFP was purified from *E. coli* strain JM109 expressing pBAD-GFP. GFP-ssrA was purified from an *E. coli clpP clpX lon* triple mutant expressing pBAD-GFP-ssrA. The absence of contaminating proteins was verified by SDS-PAGE followed by Coomassie blue staining.

***In vitro* proteolysis assays.** All *in vitro* Lon proteolysis assays were carried out with a minimal activity buffer (50 mM Tris-HCl [pH 8.0], 10 mM $MgCl_2$, and 1 mM DTT). Complete assays contained 1 μM Lon-H_6, 10 μM substrate, and an ATP regeneration system (50 mM creatine phosphate, 80 μg/mL creatine kinase [Roche], and 4 mM ATP). The reactions were incubated at 37°C and analytical samples were obtained at various time-points. The levels of untagged and tmRNA-tagged λ-CI-N at selected time-points were analyzed by Tris-tricine-

PAGE and quantified using a GS-710 imaging densitometer and Quantity One software (Bio-Rad). The levels of GFP or GFP-ssrA at selected time-points were determined from fluorescence measurements obtained using a SpectraMax M2 microplate reader (Molecular Devices) configured with empirically determined excitation and emission wavelengths of 476 nm and 519 nm, respectively.

Results

Lon Protease Functions in *trans*-Translation. To confirm that the endogenous protein tagging results I had observed for my *lon*::Tn mutants (Fig. 2.5) were due to transposon insertion in the *lon* gene and not a consequence of combined multiple integration events, I took two complementary approaches. First, I attempted to complement the *lon*::Tn λ*imm*P22 *c2-5 dis* phage phenotype with a plasmid-borne copy of the *lon* gene. I cloned *lon* flanked by its native promoter and terminator in pBR322, to generate pLon. Complementation of each of the three *lon*::Tn mutants with the pLon plasmid restored mutant sensitivity to λ*imm*P22 *c2-5 dis* phage to levels comparable to the parental wild-type strain (Fig. 3.1). Second, I reasoned that if the endogenous protein tagging phenotype of my *lon*::Tn mutants was solely due to the loss of Lon function, then an identical phenotype should be observed with an independently derived and widely used *lon* mutant (*lon*::tet). To this end, I compared the $tmRNA^{H6}$-tagging phenotype of my three *lon*::Tn mutants with that of the *lon*::tet mutant. All four *lon* mutants were found to accumulate comparably high levels of $tmRNA^{H6}$-tagged proteins

(Fig. 2.5C), suggesting that this defect was a direct result of loss of Lon protease function. Since all three of my *lon*::Tn mutants produced matching results in my assays, I chose to use one mutant, namely *lon-1*, for the further characterization of *lon* in *trans*-translation.

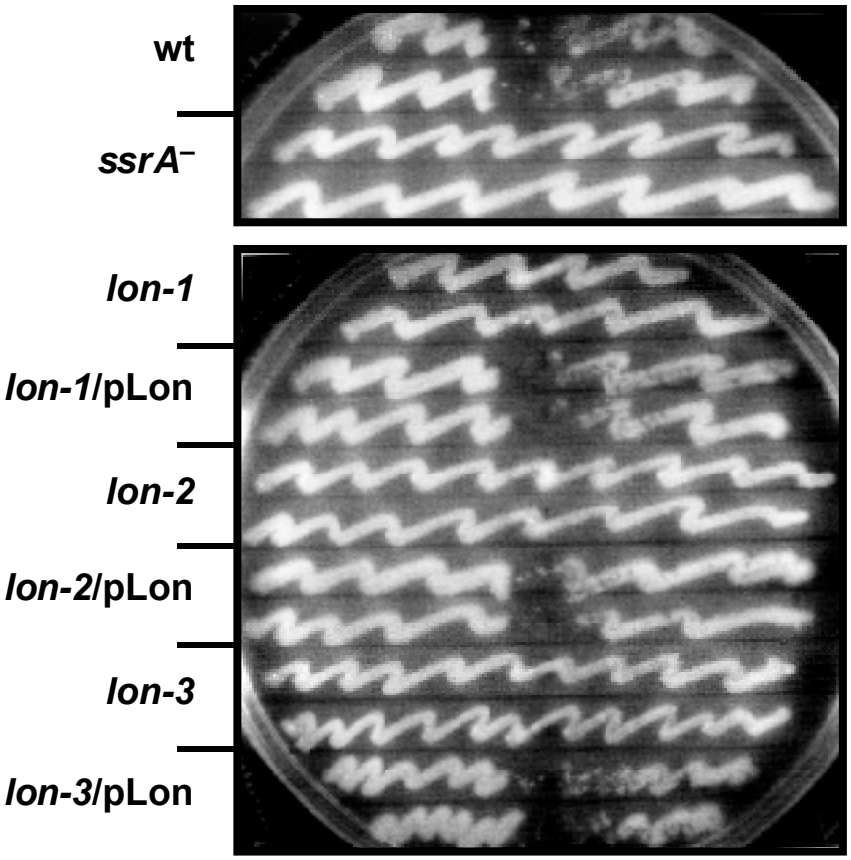

Fig. 3.1. The phage growth phenotype of *lon*::Tn mutants is complemented by plasmid pLon. The *lon*::Tn mutants *lon-1*, *lon-2*, and *lon-3* were transformed with pLon plasmid carrying the *E. coli lon* gene under the control of the native *lon* promoter. Transformants were tested for sensitivity to phage using the phage cross-streak assay. Untransformed *lon*::Tn mutants were resistant to phage, like *ssrA* mutant cells. *lon*::Tn mutants expressing pLon exhibited wild-type level sensitivity to phage. wt, wild-type.

GFP-ssrA exhibits increased stability in Lon-deficient cells. My endogenous tagging assays clearly showed that *lon* mutant cells accumulate higher levels of tmRNA^{H6}-tagged proteins. Since Lon is similar to ClpXP and ClpAP in being an ATP-dependent protease that plays an important role in cellular protein turnover, I considered whether it might also participate in the degradation of tmRNA-tagged proteins. To investigate this possibility, I determined the stability of GFP-ssrA, a GFP variant (3) that carries a hard-coded wild-type tmRNA tag (AANDENYALAA) at its C-terminus, in my *lon-1* mutant. GFP-ssrA has been used extensively to study the degradation of tmRNA-tagged proteins by the ClpXP and ClpAP proteases (17, 47, 104, 164). In wild-type cells, I found that full-length untagged GFP was quite stable throughout a two-hour time course, with a half-life of more than 120 minutes (Fig. 3.2A). In contrast, GFP-ssrA was distinctly less stable (Fig. 3.2B), exhibiting a greater than fivefold reduction in half-life to approximately 22 min. GFP-ssrA was consistently observed to be more stable in *lon-1* cells than in wild-type cells, as represented by an approximately twofold increase in reporter protein half-life (Fig. 3.2C). These data suggest that *lon* participates in the turnover of tmRNA-tagged proteins.

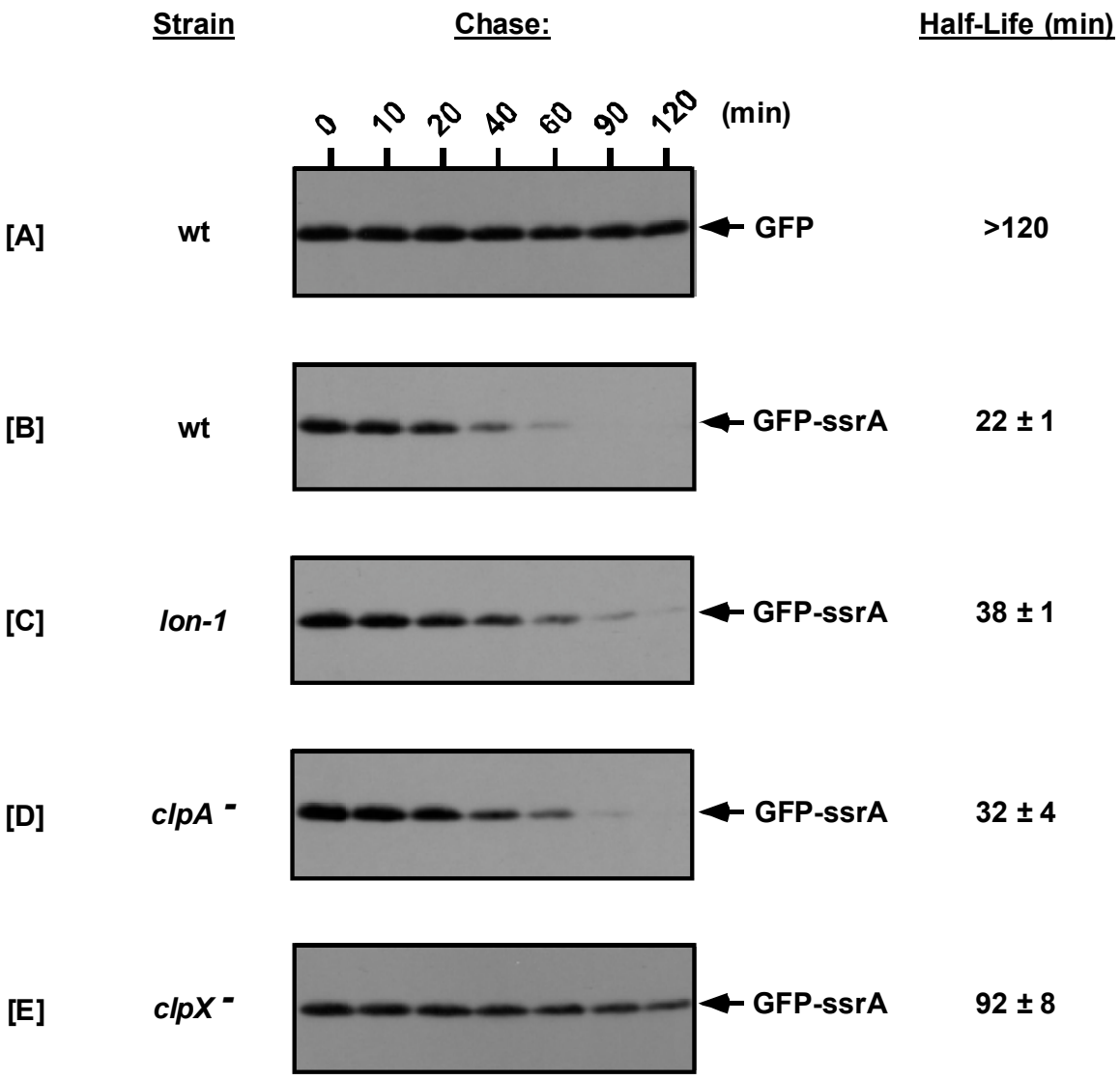

Fig. 3.2. GFP-ssrA exhibits increased stability in *lon* mutant cells. Expression of GFP or GFP-ssrA was induced using 0.01% arabinose. After removal of the inducer, protein levels were chased in medium containing spectinomycin. *In vivo* levels of GFP or GFP-ssrA were determined by SDS-PAGE and Western blot analysis using HRP-conjugated anti-GFP antibody. Parallel assays were performed with *clpA* and *clpX* mutants for comparative analysis. wt, wild-type.

To obtain comparative information for Lon and the Clp proteases, I also studied the stability of GFP-ssrA in *clpA* and *clpX* mutant cells (Fig. 3.2D-E). In each of the protease mutants, GFP-ssrA was stabilized compared to the GFP-ssrA in wild-type cells. Additionally, I observed a consistent difference in GFP-ssrA stability between *lon-1*, *clpA* mutant, and *clpX* mutant cells. As expected, GFP-ssrA was the most stable in *clpX* mutant cells (Fig. 3.2D), although it was not as stable as untagged GFP. On the other hand, GFP-ssrA was consistently more stable in *lon-1* cells than in wild-type cells or *clpA* mutant cells (Fig. 3.2C-D). My results obtained with *clpA* and *clpX* mutants are in agreement with previous reports indicating that ClpXP contributes significantly more to the *in vivo* turnover of tmRNA-tagged proteins than ClpAP contributes (17, 47, 60), and also suggest that Lon might play a greater role than ClpAP in the cellular degradation of tmRNA-tagged proteins.

λ-CI-N is efficiently tagged but more stable in Lon-deficient cells. To further substantiate my endogenous tagging and GFP-ssrA data, I decided to study a reporter that specifically activates the *trans*-translation process by causing ribosomal stalling. The *λ-cI-N-trpAt* nonstop reporter mRNA encoded by pPW500 lacks an in-frame stop codon, thus promoting ribosome stalling and cotranslational addition of the tmRNA tag to the C-terminus of the λ-CI-N-trpAt protein (hereafter referred to as λ-CI-N). Of particular relevance is the use of *λ-cI-N-trpAt* to show that the reporter protein product is tagged but stabilized in *clpP* mutants, thus directly linking Clp proteolytic activity to *trans*-translation (60).

I studied the stability of cotranslationally tagged λ-CI-N protein in wild-type, *lon-1, clpA* mutant, and *clpX* mutant cells. Consistent with my observations for GFP-ssrA, I found that cotranslationally tagged λ-CI-N was more stable in *lon-1* cells than in wild-type cells (Fig. 3.3A-B). Additionally, I found that the tagged λ-CI-N protein was most stable in *clpX* mutant cells (Fig. 3.3D), moderately stable in *lon-1* cells (Fig. 3.3B), and least stable in *clpA* mutant cells (Fig. 3.3C). In agreement with previously published reports, I found that *clpA* mutant cells have a quite mild defect in the turnover of tmRNA-tagged proteins (17, 47, 60). My findings further support my conclusion that the ATP-dependent protease Lon plays a role in the degradation of tmRNA-tagged proteins.

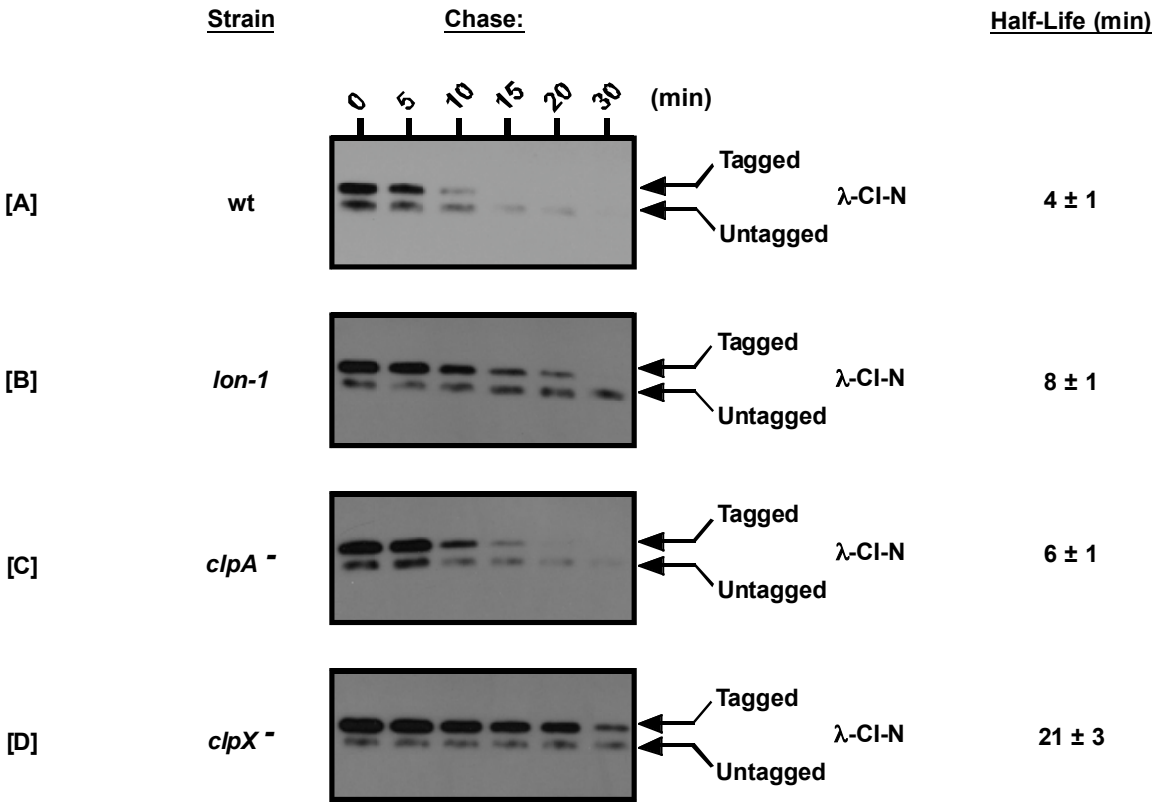

Fig. 3.3. The *trans*-translation reporter protein λ-CI-N is tagged but not efficiently degraded in *lon* mutant cells. Synthesis of the λ-CI-N protein from a nonstop mRNA activates *trans*-translation, generating tmRNA-tagged λ-CI-N. Reporter expression was induced using 1 mM IPTG. After removal of the inducer, protein levels were chased in medium containing spectinomycin. *In vivo* levels of λ-CI-N were determined by Tris-tricine-PAGE and Western blot analysis with anti-FLAG M2 (λ-CI-N has an internal FLAG M2 epitope) and anti-mouse IgG-HRP antibodies. Parallel assays were performed with *clpA* and *clpX* mutants for comparative analysis. wt, wild-type.

Highly purified Lon protease degrades tmRNA-tagged proteins *in vitro*. Although I had concurring data from three independent protein stability assays that supported a role for Lon protease in the degradation of tmRNA-tagged proteins, it was still possible that the phenotype I had observed for *lon* mutant cells resulted from some indirect effect of loss of Lon function. Lon-His_6,

the tagged and untagged forms of λ-CI-N protein, GFP, and GFP-ssrA were purified in order to study Lon selective proteolysis of tmRNA-tagged proteins. To directly examine the degradation of tmRNA-tagged proteins by Lon, I performed *in vitro* proteolysis assays with the purified Lon and the tagged and untagged forms of λ-CI-N protein (Fig. 3.4). I observed that Lon degraded tmRNA-tagged λ-CI-N protein much more efficiently than untagged λ-CI-N protein (Fig. 3.4). Previous studies had demonstrated that tagged and untagged variants of λ-CI-N are equally stable and structurally similar (60). Quantitative analysis of the degradation of tmRNA-tagged λ-CI-N confirmed that Lon protease selectively and efficiently degraded this substrate under my *in vitro* assay conditions (Fig. 3.4B). Furthermore, the *in vitro* degradation of tagged λ-CI-N was fully dependent on the presence of both Lon and ATP, as no degradation was observed in the absence of either (Fig. 3.4). These data support a model in which the tmRNA-tagged form of λ-CI-N protein is preferentially recognized and degraded by Lon in an ATP-dependent manner.

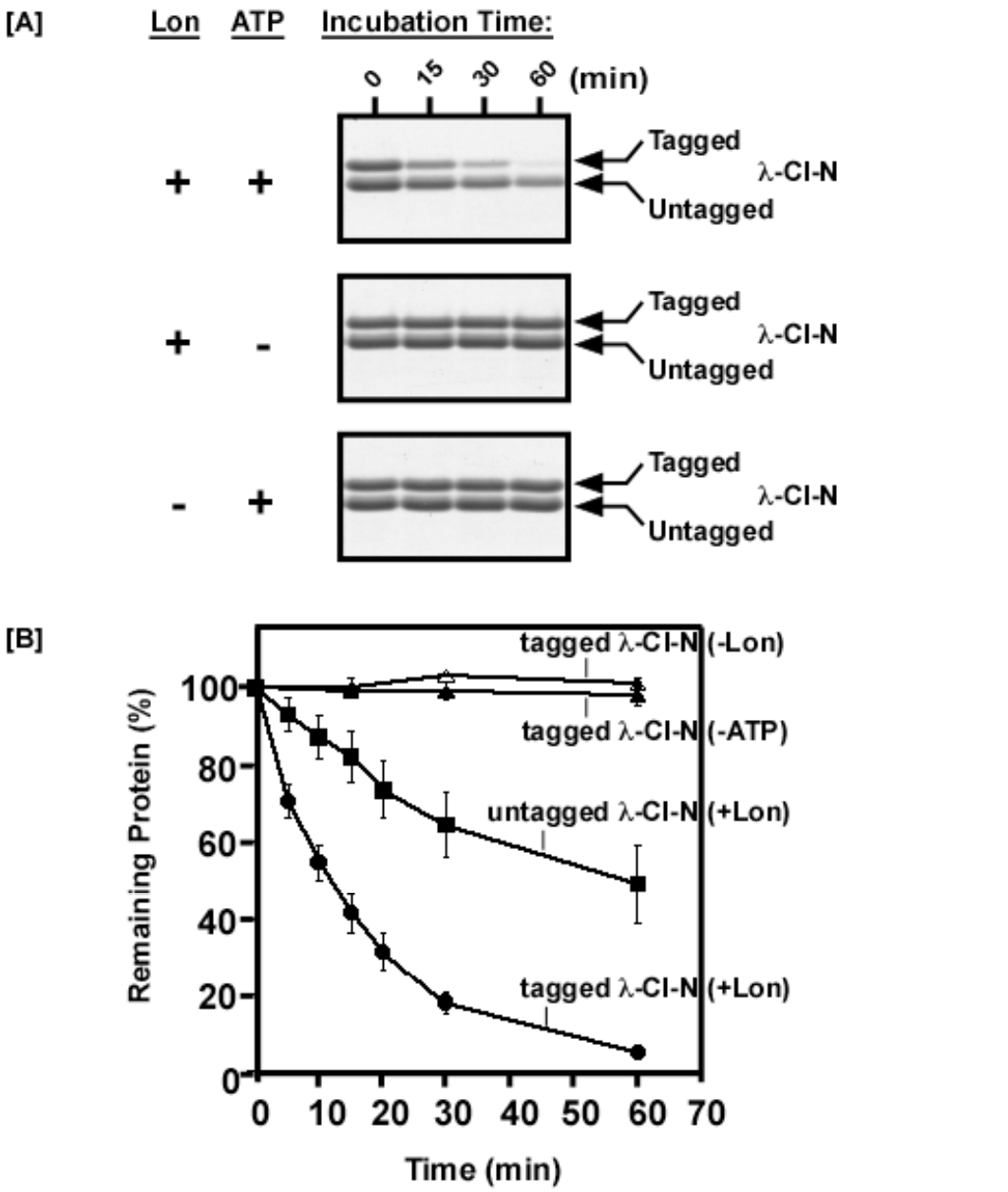

Fig. 3.4. Lon protease preferentially degrades tmRNA-tagged λ-CI-N protein *in vitro*. [A] *In vitro* proteolysis assays were carried out at 37°C in a minimal activity buffer containing 50 mM Tris-HCl (pH 8.0), 10 mM $MgCl_2$, and 1 mM dithiothreitol. Complete reactions contained 1 μM Lon, 10 μM substrate, and an ATP regeneration system. Time-point samples were taken at the indicated times and analyzed by Tris-tricine-PAGE followed by Coomassie blue staining. [B] A quantitative analysis of *in vitro* reactions performed with Lon and tagged and untagged forms of λ-CI-N is shown. Coomassie blue-stained λ-CI-N species were quantified using an imaging densitometer. Error bars represent standard deviations.

Next, I compared the proteolytic stabilities of purified GFP and GFP-ssrA in my *in vitro* degradation assay. GFP and GFP-ssrA constructs have been used extensively to examine the recognition and degradation of tmRNA-tagged proteins by the ClpXP and ClpAP proteases (18, 23, 73, 76, 90, 104, 164, 186). In agreement with previous studies and my *in vivo* results, I found that untagged GFP was highly stable and fully resistant to degradation by Lon (Fig. 3.5). The addition of the tmRNA tag to the C-terminus of GFP (GFP-ssrA) resulted in significant degradation of this protein by Lon, as measured by loss of the fluorescent signal of GFP (Fig. 3.5). Similar to my observations with tmRNA-tagged λ-CI-N, degradation of GFP-ssrA was strictly dependent on the presence of both Lon and ATP (Fig. 3.5 and data not shown). Both tagged and untagged GFP proteins were equally stable and fluorescent in the absence of Lon and ATP. These results indicated that the presence of the tmRNA tag promoted recognition and selective degradation of GFP-ssrA by Lon protease. Taken together, my data strongly support my conclusion that the ATP-dependent protease Lon participates in the cellular degradation of tmRNA-tagged proteins.

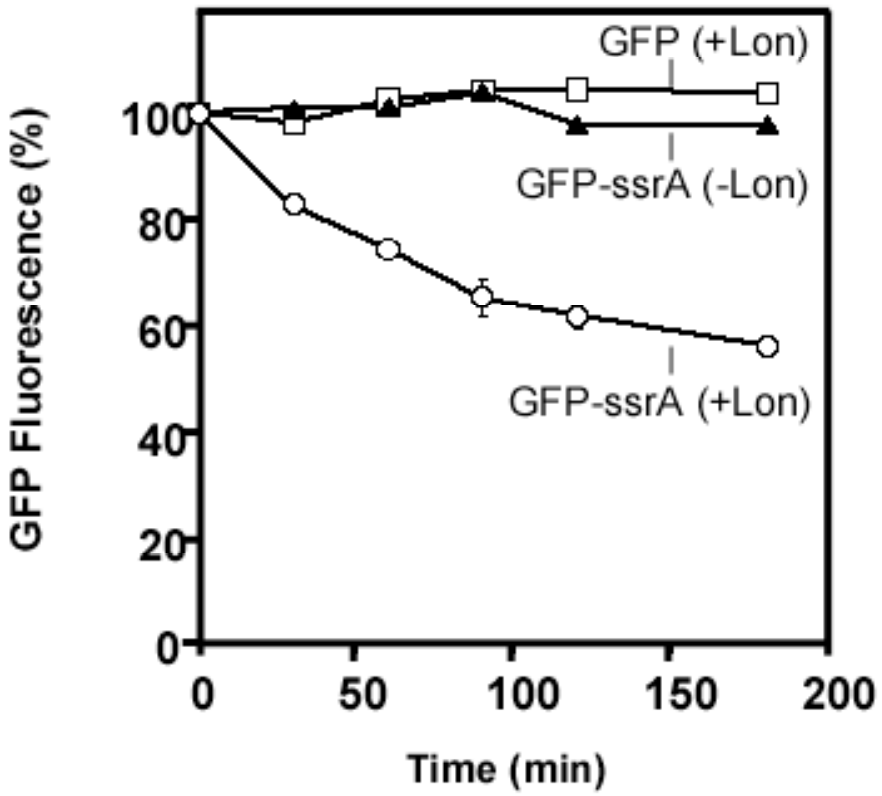

Fig. 3.5. Lon protease degrades GFP-ssrA but not GFP *in vitro*. Reactions were carried out with GFP-ssrA or GFP as described in the legend to Fig. 3.4. The levels of GFP-ssrA and GFP at various time-points were determined by microplate fluorimetry. Error bars represent standard deviations.

Discussion

While the cellular proteases ClpXP, ClpAP, FtsH, and Tsp have been shown to degrade tmRNA-tagged proteins in a tag-specific manner (60, 72, 98), this function had not been observed for Lon protease (47, 60). However, my modification of the reporter protein tagging assay for emphasis on protein decay

revealed accumulation of tmRNA-tagged λ-CI-N in *lon* mutant cells compared to wild-type cells (Fig. 3.3). Similarly, I found that the GFP-ssrA reporter protein was more stable in *lon* mutant cells than in wild-type cells. My findings are significant in demonstrating that *lon* mutants are unable to efficiently dispose of tmRNA-tagged proteins, despite possessing functional ClpXP and ClpAP proteases. Moreover, my *in vitro* degradation assays with purified components clearly demonstrated that Lon has significant proteolytic activity against tmRNA-tagged λ-CI-N and GFP-ssrA, preferentially degrading these tmRNA-tagged proteins over untagged controls.

I found that Lon exhibited more robust activity against tmRNA-tagged λ-CI-N than against GFP-ssrA *in vitro*, perhaps because GFP is intrinsically more stable than λ-CI-N. This is an interesting scenario, since *in vitro* studies have shown that this difference in inherent stability is not a complicating factor for ClpXP and ClpAP, given that the substrate carries the tmRNA tag (60, 104, 164). However, Lon is known to target certain proteins that are unstable (58, 181) and, therefore, might have more limited capacity in substrate unfolding, which is a known limitation of the ATP-dependent protease FtsH (71). Nevertheless, I have shown specific Lon activity against tmRNA-tagged proteins, indicating that the presence of the tmRNA tag stimulates proteolysis by Lon.

The C-terminal amino acids of the tmRNA tag are known to be important for substrate targeting to ClpXP and ClpAP (52). Hence, it was intriguing when comparative endogenous tagging assays performed with wild-type, *lon* mutant, *clpA* mutant, *clpX* mutant, and *clpP* mutant cells showed that while *clp* mutants

had levels of tmRNA^{H6}-tagged proteins that were similar to those observed in wild-type, *lon* mutants accumulated excessive levels of these proteins (Fig. 3.6). My data suggest that Lon might recognize sequence determinants in the N-terminal region of the tmRNA tag. There is also the possibility that the tmRNA tag aids in the presentation of Lon substrate determinants within the tagged protein, although my *in vitro* proteolysis results do argue against it.

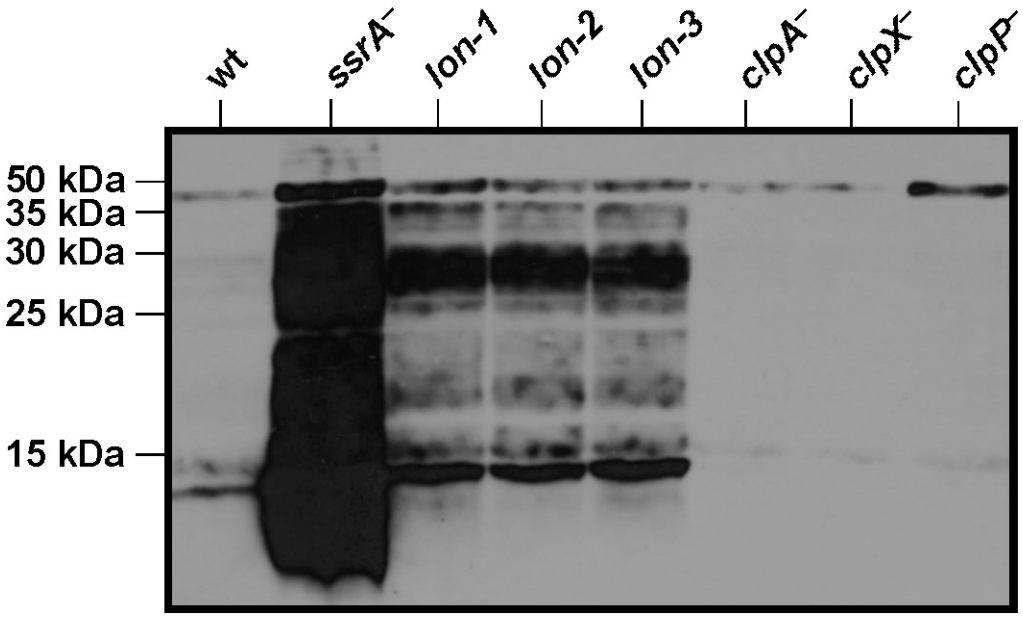

Fig. 3.6. *clp* mutant strains accumulate wild-type levels of tmRNA^{H6}-tagged proteins in endogenous tagging assays. Cells expressing plasmid-borne tmRNA^{H6} were grown in liquid culture until an OD_{600} of ≈ 1.0. Purified His_6-tagged proteins were analyzed by SDS-PAGE and detected by Western blotting using HRP-conjugated anti-His_6 antibody. The accumulation of tmRNA^{H6}-tagged proteins in *lon* mutant cells but not in *clp* mutant cells suggested that Lon is active against proteins carrying a C-terminally modified tmRNA tag. The C-terminus of the wild-type tmRNA tag carries ClpXP and ClpAP recognition determinants. wt, wild-type.

Prokaryotic proteases may exhibit overlapping substrate specificities as a compensatory mechanism to handle protease malfunction or saturation and may be less stringent in substrate selection when overexpressed (56, 89, 198). The recognition of tmRNA-tagged proteins as protease substrates may have an additional level of complexity as a function of determinants potentially present in the protein that has been modified with the tmRNA degradation sequence. Signal sequences or motifs inherent to the marked protein may affect its localization and combine with the information carried by the tmRNA tag to influence the final fate of the protein. Furthermore, degradation studies performed with the energy-dependent proteases have been limited to a select few tmRNA-tagged proteins and their derivatives (52, 60, 72), which are protein constructs that do not necessarily represent natural *trans*-translation substrates (1, 152). More extensive studies carried out with tmRNA-tagged proteins that are natural *trans*-translation or protease substrates may provide more physiologically relevant insight into substrate recognition during proteolysis.

To function both in maintenance and regulatory roles, bacterial proteases need to have rather diverse, yet defined, specificities, especially given the absence of an obligatory protein labeling system. The particular activity of a protease is also affected by its relative concentration and the activities of other proteases. In recent years, a handful of proteins that modulate the activity of bacterial energy-dependent proteases have been described (41). The ability of these adaptor proteins to influence proteolytic capacity is impressive, with observations indicating stimulatory, inhibitory, and even redirecting effects.

Of particular relevance to *trans*-translation is the key discovery that SspB, a small, ribosome-associated protein, specifically binds to the tmRNA tag of the GFP-ssrA construct and delivers it to ClpXP, thereby enhancing ClpXP degradation of GFP-ssrA (115). The action of SspB streamlines the targeting of tmRNA-tagged proteins to ClpXP and, presumably, favors the degradation of the subset of tmRNA-tagged proteins that are preferentially bound by SspB. It was found that SspB binds to a region in the tmRNA tag that overlaps with the region recognized by ClpA (52). As a result, the association of SspB with a tmRNA-tagged protein also exerts an inhibitory effect on the degradation of this protein by ClpAP.

Subsequently, an adaptor protein was discovered that exerts both stimulatory and inhibitory effects on the protease it controls. The gene encoding adaptor protein ClpS is strongly conserved as a gene immediately upstream of *clpA* in bacteria. Further analysis of *clpS* revealed that this conservation is a functional linkage, as ClpS physically associates with ClpA to modulate the degradative capacity of ClpAP (42). It was observed that ClpS inhibited the degradation of GFP-ssrA by ClpAP (42). Specifically, ClpS displaced GFP-ssrA from ClpAP by binding to the N-terminus of ClpA. The ability of an adaptor protein to specifically inhibit and divert the activity of a protease was a key finding, considering that adaptors had been generally understood to enhance the inherent degradative capacity of proteases. ClpS is also distinct from the ClpXP cofactor SspB in its ability to directly associate with the regulatory subunits of a protease as well as with protease substrates (46). Therefore, the function of ClpS

illustrates a more integrated and controlled approach to modifying protease activity. Effectively, ClpAP only degrades the proteins that ClpS allows it to.

SspB and ClpS are the only adaptor proteins known to considerably influence the proteolysis of tmRNA-tagged proteins (42, 52, 115). Collectively, both adaptors directly regulate the activities of ClpXP and ClpAP. While there is presently no known adaptor protein that specifically modulates Lon activity in tmRNA-tagged protein turnover, a few studies have shown that Lon proteolysis is influenced by cellular factors that function in stress response (109, 161). It is conceivable that Lon activity against tmRNA-tagged proteins might be stimulated by as yet unknown cellular factors. Together, the modulation of Lon, ClpXP, and ClpAP function during various physiological states and responses may lead to rearrangements in the contribution of each protease to tmRNA-tagged protein turnover. This flexibility in protease substrate recognition might be an important adaptive mechanism to environmental changes.

A longstanding unresolved issue is the fate of tmRNA-tagged proteins in bacterial species that do not possess the Clp proteases. Surveys of protease homologs and orthologs in bacteria have revealed that Lon is more strongly conserved than other bacterial energy-dependent proteases, including the Clp proteases (29, 58, 194). In contrast to the variable conservation of bacterial energy-dependent proteases, SmpB and tmRNA are strictly conserved and, presumably, are universally used to tag proteins for directed proteolysis. My finding that Lon protease participates in the cellular degradation of tmRNA-tagged proteins provides a possible resolution for this apparent paradox. The

ability of several cellular proteases to degrade tmRNA-tagged proteins suggests potentially overlapping or redundant substrate specificities and supports the significance of the proteolytic function of *trans*-translation.

In agreement with my conclusion that Lon protease participates in the degradation of tmRNA-tagged proteins, a recent unpublished study (K. McGinness and R. Sauer, personal communications) showed that Lon protease associates specifically with tmRNA and affects the stability of tmRNA-tagged RbsK protein, a natural *trans*-translation substrate. The significance of Lon-tmRNA association has not been fully elucidated and will require further scrutiny. The emerging view from these studies is that Lon protease participates in the cellular turnover of tmRNA-tagged proteins, irrespective of how they are tagged. Specifically, the substrate range of Lon includes proteins that carry a C-terminal tmRNA tag encoded at the gene level (as with GFP-ssrA), reporter proteins derived from mRNAs designed to activate *trans*-translation (as with tmRNA-tagged λ-CI-N), and proteins that are natural substrates of *trans*-translation (as with endogenously tagged proteins and RbsK). Based on the new information I have presented, I expand the current model for the degradation of tmRNA-tagged proteins to incorporate an important role for Lon protease.

Chapter 4: A Mutator Phage-Based Screen for Genes that Influence *trans*-Translation

Summary

A screen for genes that function in *trans*-translation was initiated using the temperate bacteriophage Mu. The genome of phage Mu is distinct in its ability to randomly integrate into its host's genome and, thus, may be thought of as a transposable element. Phage Mu biology bears further significance in the field of *trans*-translation, as *E. coli smpB* and *ssrA* mutants are unable to support the induction of phage Mu. Phage Mu infection was used to generate *E. coli* Mu lysogens with random genetic mutations. Mu lysogens that were unable to support the induction of Mu lytic development were then screened for resistance to phage λ*imm*P22 *c2-5 dis*, which is also dependent on *trans*-translation for lytic development. The forty final Mu lysogens were subjected to colony PCR analysis of their *smpB* and *ssrA* genes. Fifteen of the forty final Mu lysogens did not produce the expected normal PCR product, suggesting that they contained Mu prophage in *smpB* or *ssrA*. A $tmRNA^{H6}$-mediated reporter protein tagging assay showed that each of the forty final lysogens were capable of carrying out *trans*-translation and, therefore, must express functional *smpB*. Attempts to map the integration sites of Mu prophage in the final lysogens were unsuccessful, most

likely due to multiple integrations of Mu in each lysogen. The implications of the use of bacteriophage Mu for genetic screening are discussed.

Introduction

E. coli smpB and *ssrA* mutants are unable to support the growth and induction of temperate bacteriophages Mu (95, 148) and λ*imm*P22 *c2-5 dis* (95, 149). This phenotype is also observed with *Salmonella* Typhimurium *smpB* and *ssrA* mutants infected with the temperate *Salmonella* phage P22 (88, 95). While the exact reasons for phage dependence on *smpB* and *ssrA* function are not clear due to confounding studies and conflicting opinions (192), it appears that the activities of *smpB* and *ssrA* in *trans*-translation influence phage induction by modulating repressor protein function. The direct correlation of *smpB* and *ssrA* mutant phage phenotypes suggested that this common characteristic could be exploited in a screen for *E. coli* genes that are important for *smpB* and *ssrA* function (Fig. 3). Hence, a search for genes that could be functionally linked with *smpB* and *ssrA* was implemented by isolating *E. coli* mutants that could not support the induction and lytic development of Mu and λ*imm*P22 *c2-5 dis*.

Bacteriophage Mu was named after its ability to cause mutations during infection (79). While most temperate bacteriophages are specific for only one or a few integration sites in the host chromosome, Mu has the distinctive ability to randomly integrate into the host chromosome and, thus, cause random genetic mutations during lysogenization (79). Mu infection of sensitive bacteria reflects a

mutation rate of 50 to 100 times greater than spontaneous mutation frequency, and 1 to 3 percent of surviving lysogens exhibit recognizable mutations (20, 79, 180). An important characteristic of the Mu phage used in this screen is it encodes a temperature sensitive variant of the Mu C repressor that is rendered inactive at 42°C. While λ prophages can be strongly induced by the exposure of their hosts to ultraviolet light and other agents, wild-type Mu prophages are not susceptible to controlled induction and spontaneously induce at low frequencies (79). The isolation of temperature-inducible phage Mu*cts* variants (79, 185) was, therefore, a boon to Mu researchers. Mu*cts62 pAp1* (95), a temperature-sensitive variant that carries ampicillin resistance to allow for the selection of Mu lysogens, was used in a mutagenesis screen for genes that are important for *smpB* and *ssrA* function.

Materials and Methods

Bacterial strains, bacteriophages, and plasmids. Bacterial strains were cultivated in LB medium. Antibiotics (ampicillin [100 μg/uL] or kanamycin [50 μg/mL]) were added to the bacterial medium when appropriate. *Escherichia coli* K-12 derivative W3110 [F^- λ^- IN(*rrnD-rrnE*)1 *rph-1*] was utilized as wild-type and is the parental strain of the mutants analyzed in this study. Strains *ssrA::*kan (107) and Δ*smpB-1* (95) have been described previously. Bacteriophage Mu*cts62 pAp1* encodes a temperature-sensitive variant of the C repressor protein, confers resistance to ampicillin, and has been described previously (95).

λ-P22 hybrid bacteriophage λ*imm*P22 *c2-5 dis* has been described previously (169). Plasmid pPW500 (98) has a pMB1 origin of replication and encodes *λ-cI-N-trpAt* nonstop reporter mRNA regulated by a P_{TRC} promoter. Plasmid pKW510 is a derivative of pPW500 that additionally encodes wild-type tmRNA regulated by the native *ssrA* promoter. pKW540 is a derivative of pKW510 encoding variant $tmRNA^{H6}$ rather than wild-type tmRNA. The $tmRNA^{H6}$ variant encodes a modified degradation tag (NH_2-[A]ANDEHHHHHH-COOH) that is poorly recognized by proteases, in contrast to the wild-type tag (NH_2-[A]ANDENYALAA-COOH). Plasmid pKW550 is a derivative of pKW540 that confers resistance to kanamycin rather than to ampicillin.

Generation of *E. coli* Mu lysogens, phage screening, and colony PCR analysis. Performed by Dr. Wali Karzai. *E. coli* W3110 was infected with phage Mu*cts62 pAp1* in liquid culture and Mu lysogens were selected for on medium containing ampicillin. Lysogens were screened for the inability to support the induction and lytic development of phage Mu*cts62 pAp1* at 42°C. Lysogens defective in supporting the induction of phage Mu were further screened for the inability to support the lytic development of phage λ*imm*P22 *c2-5 dis* in the phage cross-streak assay described in Ch. 2. Colony PCR reactions were carried out using primers designed to amplify a 1 kb region of the *E. coli* chromosome containing *smpB* and *ssrA*. PCR products were analyzed based on electrophoretic mobility through agarose gel (1%).

$tmRNA^{H6}$-mediated λ-CI-N reporter tagging assays. Strains expressing pKW550 were cultivated in LB medium containing kanamycin. Expression of the

λ-cI-N-trpAt nonstop reporter mRNA from pKW550 was induced in cultures at an OD_{600} of ≈ 0.5 using 1 mM IPTG for 1 h. Harvested cells were lysed by resuspension and boiling in 1X Tris-tricine sample buffer. Analytical total cellular protein samples were normalized based on protein concentration (Bio-Rad Protein Assay) and resolved by Tris-tricine-PAGE. tmRNA-tagged and untagged λ-CI-N proteins were detected by Western blotting with HRP-conjugated mouse monoclonal anti-His_6 antibody (Santa Cruz Biotechnology).

Integration site mapping of Mu*cts62 pAp1* lysogens. Genomic DNA was isolated from the forty final candidates using the QIAamp DNA Mini kit (Qiagen). Candidate genomic DNA samples were used as templates for DNA sequencing reactions (Stony Brook DNA Sequencing Facility) containing various primers specific to either end of the Mu*cts62 pAp1* genome (61, 129).

Results

A Mutator phage-based screen for genes that function in *trans*-translation. E. coli W3110 was infected with a temperature-inducible Mu bacteriophage to generate Mu lysogens with random genetic mutations. Since Mu lytic development cannot be induced in cells that are defective in tmRNA and SmpB function, Mu lysogens that were unable to support Mu induction at 42°C were retained as initial candidates. These initial candidates were then assayed for the inability to support the growth of hybrid phage λ*imm*P22 *c2-5 dis* (95, 169). λ*imm*P22 *dis c*2-5 is one of several hybrid phages that can be generated

from the homologous recombination of *E. coli* phage λ and *Salmonella* Typhimurium phage P22 (22, 55, 200). λ*imm*P22 hybrids that express the P22 wild-type *c1* gene, which encodes a protein that promotes lysogeny and controls the transcription of the *c2* repressor gene, are unable to grow in *E. coli smpB* and *ssrA* mutants (95, 149). The use of both Mu and λ*imm*P22 *c2-5 dis* phages was designed to favor the isolation of Mu lysogens that were defective in *trans*-translation. The uninducible Mu lysogens that were unable to support the lytic development of λ*imm*P22 *c*2-5 *dis* phage were retained as final candidates (Fig. 4.1A-B).

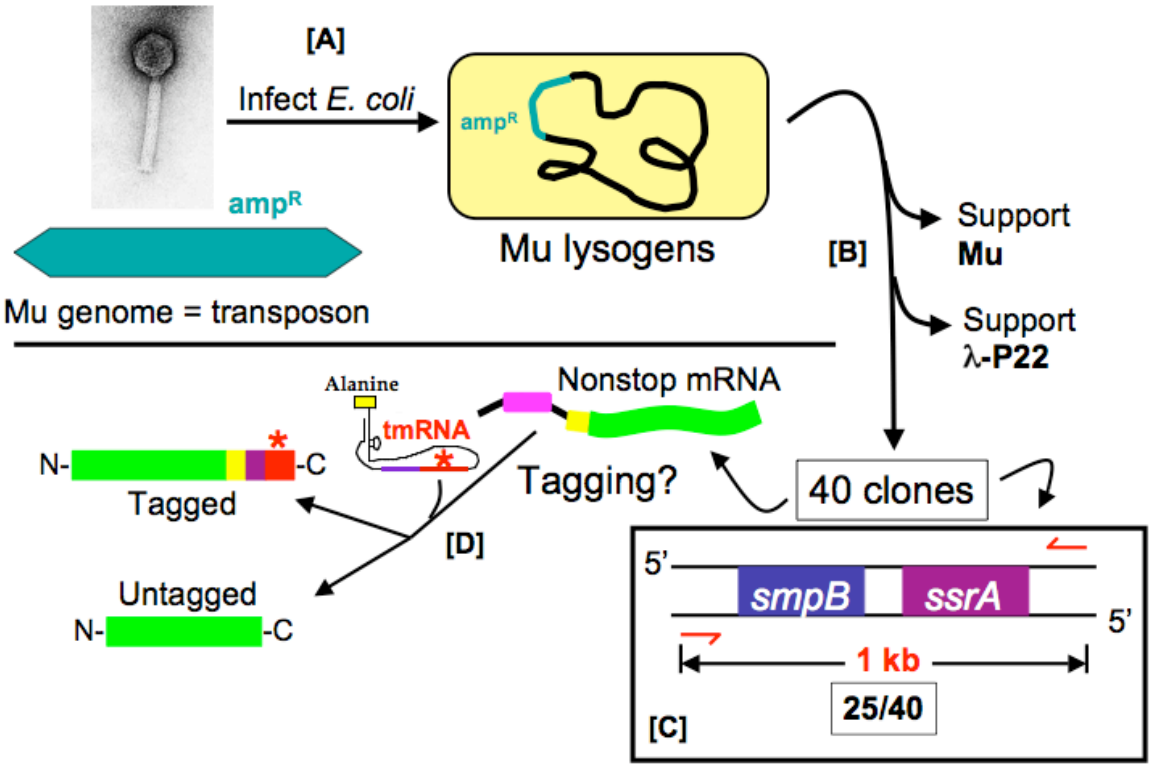

Fig. 4.1. A Mutator phage-based screen for genes that function in *trans*-translation. *E. coli* mutants that lack *smpB* or *ssrA* do not support the induction of phage Mu or the lytic development of phage λ*imm*P22 *c2-5 dis*. [A] *E. coli* wild-type strain W3110 was infected with phage Mu to generate Mu lysogens. [B] To exclude genes that are not important for *smpB* or *ssrA* function, Mu lysogens that supported phage Mu induction (assayed at 42°C) were removed from this screen. Subsequently, uninducible Mu lysogens that supported the lytic development of λ*imm*P22 *c2-5 dis* were removed from this screen, leaving 40 final candidates. [C] During colony PCR analysis using primers designed to amplify a 1 kb region spanning *smpB* and *ssrA*, 25 of the 40 final candidates produced a 1 kb product and were expected to contain normal *smpB* and *ssrA*. The remaining 15 clones did not produce a 1 kb product and were expected to contain prophage in *smpB* or *ssrA*. [D] All of the 40 final candidates were then tested for *trans*-translation activity in a $tmRNA^{H6}$-mediated reporter protein tagging assay.

Colony PCR analysis. Forty Mu clones that were unable to support the induction of Mu and unable to support the lytic development of λ*imm*P22 *c2-5 dis* were isolated from this screen. Given that *smpB* and *ssrA* are immediately adjacent to each other in the *E. coli* chromosome, colony PCR analysis using primers complementary to the sequences upstream of *smpB* or downstream of *ssrA* was carried out to study *smpB* and *ssrA* in each of the forty final candidates. A lysogen with uninterrupted *smpB* and *ssrA* was expected to produce a 1 kb PCR product, which was the case for twenty-five of the forty final lysogens (Fig. 4.1C). The remaining fifteen lysogens did not produce 1 kb PCR products and were expected to contain Mu prophage integrated in *smpB* or *ssrA*. Note that the Mu genome is very large (Fig. 4.2) and, therefore, a correspondingly higher molecular weight PCR product was not expected from lysogens carrying Mu in *smpB* or *ssrA*.

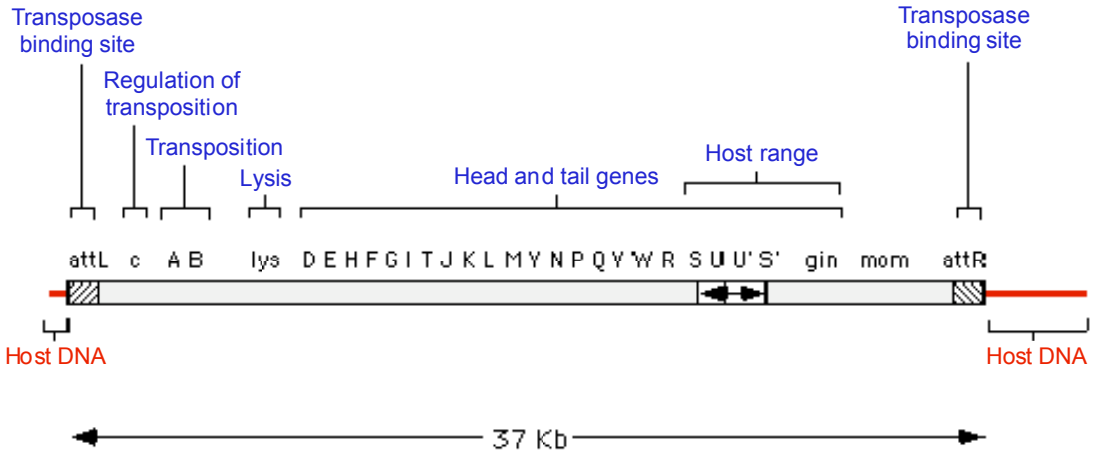

Fig. 4.2. A simplified schematic of the phage Mu genome. Primers for Mu prophage integration site mapping in the forty final candidates were designed based on the left (attL) and right (attR) sequences of the Mu genome. Figure from (121).

Assessment of *trans*-translation in the final candidates. To characterize the forty final candidates based on a known *smpB* and *ssrA* function, they were assayed for the ability to tag an exogenous substrate known to activate *trans*-translation (Fig. 4.3). This substrate was a plasmid-encoded, IPTG-inducible *λ-cI-N-trpAt* nonstop mRNA. This mRNA reporter construct directs the synthesis of the N-terminal domain of the λ repressor protein (designated λ-CI-N) and lacks an in-frame stop codon (98). The *λ-cI-N-trpAt* mRNA was designed to mimic a truncated or nonstop mRNA and, thus, be an activator of ribosomal stalling and *trans*-translation. To facilitate reporter protein detection and purification, the λ-CI-N protein contains internal FLAG M2 and His_6 epitopes. $tmRNA^{H6}$, a tag variant of tmRNA, was used in this assay to report tmRNA-mediated tagging of the λ-CI-N protein (Fig. 4.3). $tmRNA^{H6}$ encodes a

modified degradation tag that is stabilized compared to the wild-type tmRNA degradation tag. Proteins that are modified with the tmRNA^{H6} tag may be purified using Ni^{2+}-NTA chromatography and detected by Western blotting analysis using anti-His_6 antibody.

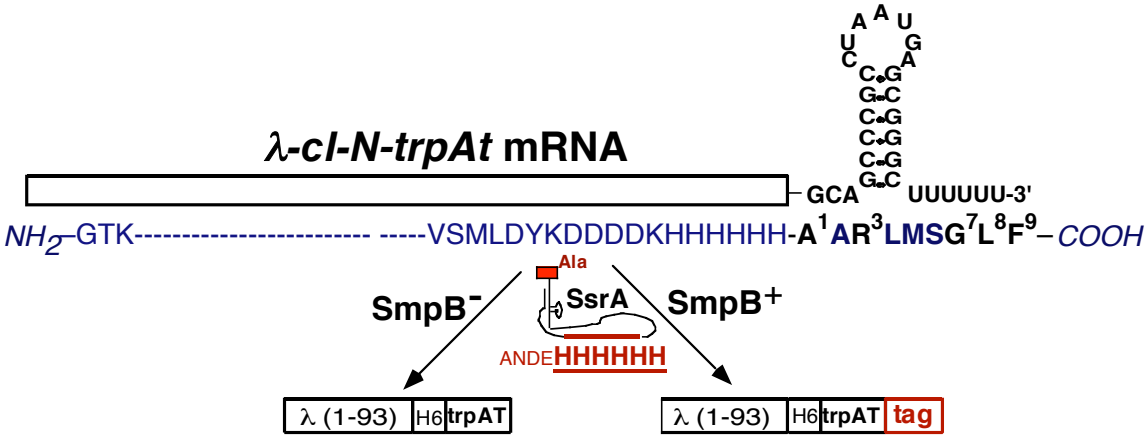

Fig. 4.3. The λ-CI-N reporter protein tagging assay. The *λ-cI-N-trpAt* nonstop mRNA encodes the N-terminal domain of the λ repressor protein fused to FLAG M2 and His_6 epitopes and ends without an in-frame stop codon. The λ-CI-N protein encoded by *λ-cI-N-trpAt* is shown directly underneath the mRNA sequence. Variant tmRNA^{H6} was used in this assay to facilitate the stabilization and detection of tagged λ-CI-N protein. Strains lacking SmpB were expected to accumulate only untagged λ-CI-N protein, whereas strains expressing functional SmpB were expected to produce tmRNA^{H6}-tagged λ-CI-N protein.

Based on the colony PCR results, it was expected that at least some of the forty final candidates would be defective in tmRNA-mediated protein tagging due to *smpB* or *ssrA* disruption by Mu prophage. Notably, the tmRNA^{H6} variant used in my tagging assays had the potential to rescue the defective tagging phenotype of an *ssrA*::Mu mutant and it was understood that, in contrast to an *smpB*::Mu mutant, an *ssrA*::Mu mutant should be able to support tmRNA^{H6}-mediated tagging of λ-CI-N in my tagging assays. Therefore, lysogens

expressing functional *smpB* were expected to accumulate mainly tagged λ-CI-N protein and lysogens containing defective *smpB* were expected to accumulate only untagged λ-CI-N protein, whereas lysogens containing defective endogenous *ssrA* were expected to exhibit a tagging profile similar to that of an *ssrA* mutant control (Fig. 4.4, Δ*A* lanes). Two independent tagging assays with the forty final candidates clearly showed that they were each able to tag the λ-CI-N protein using tmRNA^{H6} (Fig. 4.4). The tmRNA^{H6}-mediated λ-CI-N protein tagging assays indicated that each of the forty candidates expressed partially or fully functional SmpB, while PCR analysis suggested that some fraction of them should be fully defective in SmpB.

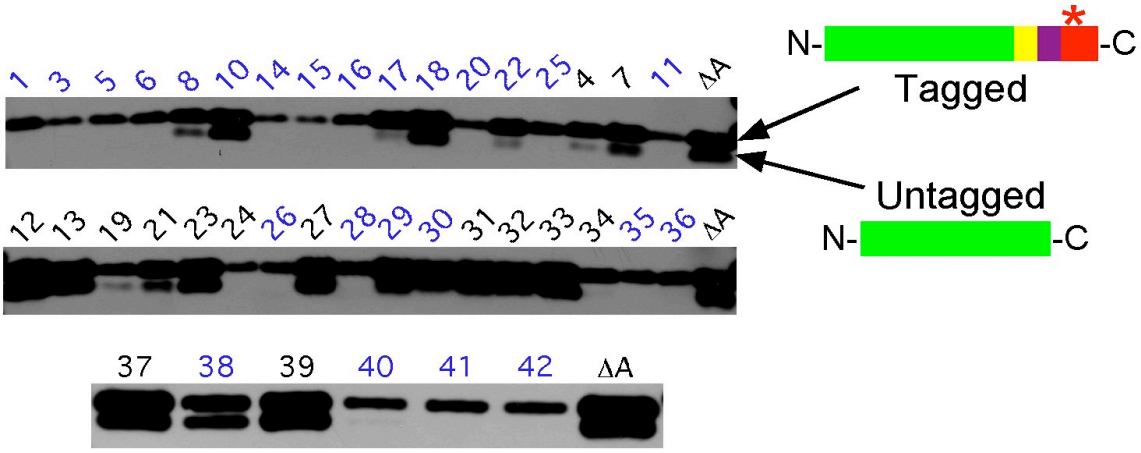

Fig. 4.4. tmRNA^{H6}-mediated λ-CI-N tagging assays performed with final screen candidates. The 40 final candidates and a control *ssrA* mutant (ΔA) were transformed with a plasmid encoding the λ-CI-N construct and tmRNA^{H6}. Reporter expression was induced using 1 mM IPTG. *In vivo* levels of tagged and untagged λ-CI-N were determined by Tris-tricine-PAGE and Western blotting analysis using anti-His$_6$-HRP antibody. All of the 40 final Mu lysogens supported tmRNA^{H6}-mediated tagging of λ-CI-N via *trans*-translation. The screen candidates were named numerically. Clones 1, 3, 5, 6, 8, 10, 11, 14-18, 20, 22, 25, 26, 28-30, 35, 36, 38, and 40-42 were suggested by colony PCR analysis to contain wild-type *smpB* and *ssrA*.

The levels of tmRNA^{H6}-tagged λ-CI-N protein that accumulated in the forty final lysogens were variable. Some clones had similar, slightly reduced, or significantly reduced levels of tagged λ-CI-N compared to the *ssrA* mutant control. Untagged λ-CI-N and tmRNA^{H6}-tagged λ-CI-N accumulated to comparable levels in *ssrA* mutant cells (Fig. 4.4, Δ*A* lanes). This pattern was observed for some of the lysogens, but untagged λ-CI-N was detected as the minor species in or was completely absent from many of the lysogens, suggesting possible endogenous tmRNA activity in those clones. The three types of patterns described above were observed in the group of twenty-five lysogens suggested by PCR analysis to contain wild-type *smpB* and *ssrA*, as well as in the remaining lysogens.

Endogenous tmRNA might counteract tmRNA^{H6} by contributing to the tagging of λ-CI-N during the tagging assay. λ-CI-N protein tagged with the wild-type tmRNA-encoded proteolysis tag should be quickly degraded. Hence, the tmRNA^{H6}-mediated tagging profile of mutants expressing functional *ssrA* might be reduced compared to that of an *ssrA::Mu* mutant. This possibility might account for the sole and reduced accumulation of tmRNA^{H6}-tagged λ-CI-N in some mutants relative to the levels accumulated in the *ssrA* mutant control. An *smpB* mutant control was consistently found to be defective in tmRNA^{H6}-mediated tagging of λ-CI-N (data not shown), as expected, due to the absence of functional *smpB*. Possible reasons for the large accumulation of tagged λ-CI-N in some of the twenty-five clones suggested by colony PCR analysis to have wild-type *smpB* and *ssrA* is Mu prophage inactivation of genes that influence the

proteolytic degradation of proteins tagged via *trans*-translation and Mu prophage inactivation of genes that influence the maturation of tmRNA. The fact that each of the forty final lysogens was able to support $tmRNA^{H6}$-mediated tagging of λ-CI-N protein also conveys the possibility that some of the interrupted genes in these lysogens are important for Mu induction and λ*imm*P22 *c2-5 dis* lytic development, rather than for *smpB* and *ssrA* function.

Mu prophage integration site mapping. The next step of this screen was to identify the host sequences interrupted by Mu prophage. DNA primers were designed for complementary annealing to the left or right end of the Mu genome (Fig. 4.2). These primers should direct PCR extension from the ends of a prophage toward the surrounding host DNA and, therefore, allow identification of the prophage integration site in each of the forty final candidates. Unfortunately, it seemed that the inherent simplicity of this procedure was marred by the complexity of phage Mu (79, 176). Numerous sequencing reactions produced a similar result: initially strong, clean sequencing data that immediately became noisy and unreadable once the Mu-specific sequences ended. While I had initially thought that suboptimal PCR conditions or stable secondary structures of the genomic DNA templates were contributing to the degeneration of the sequencing data, it soon became apparent that the signals corresponding to Mu prophage sequences were often dramatically amplified compared to the weaker and erratic signals they quickly deteriorated into. An alternative explanation for my sequencing results is that they were actually reflecting the genetic nature of my candidates. The presence of two or more Mu prophages in

a given lysogen should produce enhanced sequence data from the Mu genome and the simultaneous sequencing of the surrounding host sequences at two or more different integration sites should lead to signal interference during the data readout. The results of several DNA sequencing attempts with primers designed to anneal to different regions in the ends of Mu support my inference of polylysogeny. Successful mapping of the Mu integration sites in each polylysogen will require effective separation of the multiple prophages. Phage P1 transduction techniques may be considered for this purpose.

Discussion

The developmental requirements of bacteriophages Mu and λ*imm*P22 *c2-5 dis* were used in a screen to identify genes associated with *smpB* and *ssrA* function. The initial screening for uninducible Mu clones should have isolated lysogens with mutations in genes that directly inflence *trans*-translation, as well as lysogens with mutations in genes that influence Mu induction but not *trans*-translation. The subsequent screening of the uninducible Mu lysogens for the inability to support λ*imm*P22 *c2-5 dis* lytic development was carried out based on the overlapping growth defects of Mu and λ*imm*P22 *c2-5 dis* in *E. coli smpB* and *ssrA* mutants. However, screening for mutant resistance to λ*imm*P22 *c2-5 dis* phage did not support the proper assessment of overall mutant susceptibility to phage infection. Therefore, the nature of this assay did not specifically eliminate Mu lysogens that were generally resistant to phage infection in favor of retaining lysogens that were defective in *trans*-translation. This caveat of the screen may account for the ability of each final candidate to tag the λ-CI-N reporter via *trans*-translation.

The design of this screen was based on the premise that uninducible Mu clones that are also unable to support the lytic development of λ*imm*P22 *c2-5 dis* are more likely to contain defects in genes that are functionally related to *smpB* and *ssrA*. The use of λ*imm*P22 *c2-5 dis* in this screen was intended to facilitate the exclusion of genes that are important for Mu induction but unrelated to *smpB* and *ssrA* function. However, *E. coli* genes that function independently of *trans*-

translation and are required for the growth of both Mu and λ*imm*P22 *c2-5 dis*, or are required for attachment and infection by λ*imm*P22 *c2-5 dis*, may have been retained in the screen. Additionally, genes that are important for possible *smpB* and *ssrA* functions that do not influence Mu and λ*imm*P22 *c2-5 dis* growth may have been screened out. The clear identification of *smpB* or *ssrA* mutants, generated by the integration of Mu into *smpB* or *ssrA,* would help confirm the validity of this screen.

Ultimately, the host sequences interrupted by Mu prophage could not be identified using the sequencing method I have described. I feel that the most likely explanation for my sequencing results is Mu polylysogeny. There remains the possibility of using phage P1 transduction to separate the prophages from each polylysogen for subsequent integration site mapping. However, a troubling consequence of polylysogeny is its impact on the validity of this mutagenesis screen. An oversight in the design of this screen was the molecular biology of phage Mu. High titers of phage Mu during infection promotes the establishment of polylysogeny and during phage DNA replication, Mu generates multiple copies of its genome through dispersed transposition into the host chromosome (79, 176). As I have shown in this chapter, the generation of Mu polylysogens in a genetic screen presents important challenges during the characterization and identification of screen candidates. It seems that phage Mu mutagenesis might be best applied to the mutation or inactivation of a known target gene or best performed with a modified version of Mu that does not polylysogenize or that has limited transposition capacity.

Polylysogeny during the Mu infection step of this screen is likely to be more problematic than the establishment of polylysogeny at later steps. Early polylysogeny might undermine the reliability of the screening procedure by introducing variables that are subject to screening or that reduce mutant viability. A Mu polylysogen might be hypersensitive to the elevated temperature used to identify uninducible clones or might be simply inviable as a result of multiple Mu insertions. Mu polylysogens with prophages inserted into any genes that are important for *smpB* or *ssrA* function and into any essential genes would not be isolated in the screen I have described. Polylysogens with multiple gene mutations may also be more likely to exhibit epistatic phenotypes that are not consistent with the phenotypes associated with single gene mutations, especially if one mutation happens to conceal or rescue another mutation. Additionally, the formation of stable Mu lysogens is inefficient, with only 5 to 10 percent of the infected cells surviving as lysogens (79). The combination of possible polylysogeny and inefficient lysogenization suggests that many relevant lysogens may have been lost from or obscured in this screen due to inviability, epistasis, or phage Mu lytic development.

Aside from the polylysogeny problem, certain features of *trans*-translation may still be investigated in the final candidates. The *ssrA* gene of each Mu lysogen may be more accurately studied through DNA sequencing analysis, and these results can then be compared with the results from the colony PCR analysis and the λ-CI-N protein tagging assays in order to obtain a better grasp of the nature of the mutations in the final candidates. Northern blotting analysis of

ssrA may also be carried out to investigate the expression and processing of tmRNA in the candidates.

Chapter 5: Concluding Remarks and Future Directions

Screening for Genes that Function in *trans*-Translation

In this dissertation, I have described my efforts to use genetic screens for identifying genes that are important for *trans*-translation. There are clearly some key limiting factors associated with genetic screens, such as a high likelihood of not isolating genes that are essential for organism viability and the need to have large sampling sizes, but the potential for discovery and the straightforwardness associated with genetic screens serve to consistently rationalize their use. Indeed, the favorable characteristics of genetic screens are emphasized in strongly engineered approaches such as the yeast two-hybrid system, which invoke a guilty-by-association rationale and circumvent the physiological consequences of mutagenesis.

Despite the potential for success and the high regard bestowed upon functional screening methods, the reward of positive results is not guaranteed. In fact, the possibility of discovering nothing new about an organism or a pathway of interest is high enough to garner such a proposal strong criticism and wary regard. Reflecting on the outcomes of my attempts to further characterize *trans*-translation by searching out novel functional associations and on the extent of our current understanding of *trans*-translation [(44) and Fig. 5.1], it seems that

my efforts did carry a significant risk of not producing something new to be characterized.

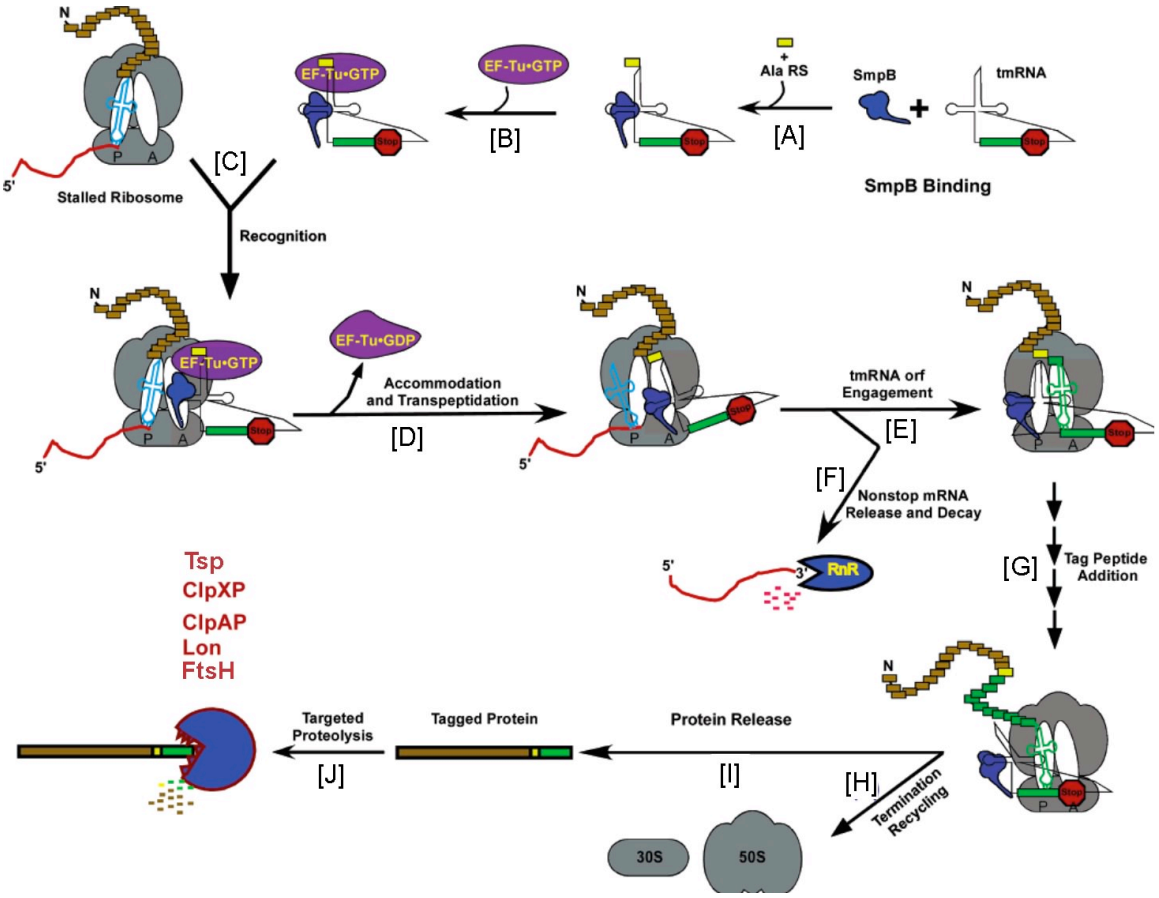

Fig. 5.1. Integrated model of *trans*-Translation. Additional features of *trans*-translation have been determined since the proposal of the original model (98). [A] SmpB enhances the alanyl-charging of tmRNA by alanyl-tRNA synthetase. [B] SmpB and alanyl-charged tmRNA form a quaternary complex with EF-Tu•GTP, which is required for [C] the delivery of an SmpB•tmRNA complex to a ribosome that has stalled on an aberrant mRNA. [D] Proper SmpB•tmRNA accommodation into the empty A site of a stalled ribosome leads to transpeptidation of the nascent peptide from the P site tRNA onto the tRNA-like domain of tmRNA. [E] The ribosome then engages on the mRNA-like domain of tmRNA. [F] The cotranslational switching of templates results in the release of the aberrant mRNA, which is degraded in an RNase R-dependent manner. [G] Translation of the tmRNA ORF results in the addition of the tmRNA tag to the C-terminus of the nascent peptide. Translation terminates at the stop codon that marks the end of the tmRNA ORF, releasing [H] recyclable ribosomal subunits and [I] the tagged protein. [J] The tagged protein is subjected to targeted proteolysis by the proteases Tsp, ClpXP, ClpAP, Lon, and FtsH. Figure adapted from (44).

It is not that *trans*-translation is a trivial pathway. It is that *trans*-translation seems to share components with cellular mechanisms that are even more critical for cell viability. Perhaps that is the best strategy for a biological quality control pathway, especially if the execution of the pathway serves to reflect the overall fitness of the cell. Perhaps the unavailability of certain critical RNAs or proteins would negate the purpose of even carrying out a quality control pathway. In any case, the ability of the tmRNA and SmpB to mediate *trans*-translation is directly dependent on cellular factors that are dedicated to translation or degradation processes. Given such circumstances, the chances of identifying a completely novel *trans*-translation factor might be quite low. The activities of the bifunctional tmRNA, its dedicated protein cofactor SmpB, and an assortment of translation factors, RNases, and proteases with unrealized capacities might more than satisfy the requirements for *trans*-translation.

My bacteriophage screening of approximately 19,000 *E. coli* transposon insertion mutants for *trans*-translation defects concluded with 9 candidate genes, aside from *smpB* and *ssrA* (Ch. 2). While I have successfully shown that my strongest gene candidate *lon* functions in *trans*-translation (Ch. 3), there remains the possibility that the other candidate genes are also important for *trans*-translation. Endogenous protein tagging assays performed with the remaining mutants showed that they were mostly normal in the tagging function of *trans*-translation. Since the endogenous protein tagging assay involves the use of a plasmid-borne tmRNA that encodes a stabilizing peptide tag ($tmRNA^{H6}$), mild or moderate defects in *trans*-translation would be difficult to determine in this assay.

Defects in the remaining candidate genes might negatively affect the expression or function of endogenous tmRNA or the regulation of translation or degradation processes. Thus, the use of other assays is needed to further investigate *trans*-translation activity in the remaining candidates. Additional characterization of the remaining secondary candidates should include the individual evaluation of tmRNA and SmpB expression, function, and ribosome association. The failure of additional *trans*-translation specific assays to link these remaining candidate genes to *trans*-translation function would suggest that these genes are screen artifacts resulting from bacteriophage screening. Phage dependence on tmRNA and SmpB has been shown to be due to a requirement for *trans*-translation (191), so there is only a small possibility that the remaining secondary candidates are linked to tmRNA and SmpB through an unrelated pathway.

The identification of *lon* in my transposon mutagenesis-based screen for genes that function in *trans*-translation seems particularly fortuitous, considering that I did not identify the genes for proteases that were already known to degrade tmRNA-tagged proteins *in vivo* (60, 72, 98). In fact, the sensitivity of *clpA*, *clpX*, and *clpP* mutants to lysis by the initial screening phage λ*imm*P22 *c2-5 dis* suggests that this phage does not require the full capacity of *trans*-translation to support its biology. The implication of this selective dependence is that my use of phage λ*imm*P22 *c2-5 dis* could not have been able to yield a complete list of non-essential genes that support *trans*-translation. The success and realization of the other screening methods I have worked on (Ch. 4 and below) would have

been particularly useful for the evaluation of my transposon mutagenesis screen and, potentially, for the identification of other key *trans*-translation components.

Using the Yeast Two-Hybrid System to Identify Protein-Protein Interactions in *trans*-Translation

The yeast two-hybrid system was developed as a method for readily detecting the physical association of two proteins *in vivo* (33, 50). While the yeast two-hybrid system operates within the yeast cell nucleus, an environment that can be especially problematic for the investigation of membrane or membrane-associated proteins, this technique has proven to be invaluable for the study of protein function in organisms ranging from bacteria to humans. The yeast two-hybrid system represents an excellent starting point for understanding the interplay between proteins that define physiological processes. Additionally, the *in vivo* setting and the straightforward signal detection have lent the system to various modifications designed to improve screening sensitivity and efficiency (141, 168).

The basic requirements for a yeast two-hybrid assay include an appropriate yeast strain to perform the assay in, two types of hybrid proteins, and a method of detecting fusion protein interaction. One of the hybrid proteins consists of the DNA binding domain of a transcription factor fused to a protein of interest. This chimera is the designated bait protein and is the protein that one hopes to characterize through the identification of other proteins that interact with

it. The second fusion protein consists of the activation domain of a transcription factor fused to a protein that possibly interacts with the bait protein of interest. This chimera is considered the prey protein and its exact identity may or may not be known by the researcher. The simplest yeast two-hybrid assays involve defined bait and prey hybrids that have been specifically designed for interaction analysis, while more comprehensive assays involve prey libraries built from a species' genome or involve multiple baits arrayed with different prey libraries. High-throughput yeast two-hybrid screens that are designed to determine interactomes, or protein-protein interactions for a given species or complex pathway, utilize bait and prey matrix approaches that are under constant development to increase sensitivity and sampling efficiency (141, 168).

I have generated a set of highly representative *Yersinia pestis* genomic DNA activation domain libraries to be used in yeast two-hybrid screening. These libraries were prepared for the purpose of screening for proteins that interact with SmpB and tmRNA, but have general applicability to screens designed to identify other interactions as well. *Y. pestis* is the etiological agent of plague and was chosen as the framework for yeast two-hybrid study with the objective of characterizing *trans*-translation function in a potential biowarfare instrument. tmRNA and SmpB have been demonstrated to be important determinants of pathogenicity in several bacteria (14, 88, 92), including in *Yersinia pseudotuberculosis* (137).

My activation domain fusion libraries were generated based on the method described by James et al. (85). These libraries were prepared using the

three activation domain vectors pGAD-C1, pGAD-C2, and pGAD-C3 (85), each of which represents one of three translational reading frames. A highly representative yeast two-hybrid library includes several different fusion points to maximize the potential for relevant interactions (85). Such a library can be readily generated by using various restriction endonucleases (REases) to digest genomic DNA. These REases should each have dissimilar recognition sequences, but all produce DNA fragments with 5'-CG-3' overhangs that can be cloned into the unique *Cla* I restriction site in the activation domain vectors.

A collection of nine 5'-CG-3' REases (obtained from New England Biolabs) was used in preliminary tests to determine if each enzyme could cleave *Y. pestis* genomic DNA with high enough frequency to generate fragments ranging from 500 bp to 3000 bp (Fig. 5.2). Three enzymes were observed to cleave the DNA with very low frequencies at concentrations of up to 25 units of enzyme per μg of DNA, and were excluded. Five of the remaining six enzymes cleaved the DNA with high frequencies, and the last enzyme exhibited a somewhat lower cleavage frequency. The five efficient enzymes (TaqI, HpyCH4IV, MspI, AciI, and HinP1I) are each specific for a single tetrameric sequence. The sixth, moderately efficient enzyme (BsaHI) recognizes multiple hexameric sequences that vary at two base positions. The three excluded enzymes (BspDI, BstBI, and NarI) each recognize a single hexameric sequence and likely exhibited a more limited digestion pattern due to the lower occurrence of such sequences. Based on the restriction patterns observed for the six enzymes that passed my preliminary testing, 500 bp – 3000 bp (TaqI, HpyCH4IV,

MspI, AciI, and HinP1I) or 500 bp – 4000 bp (BsaHI) genomic DNA fragments were purified from each of the REase digests. The five sets of restriction fragments were separately cloned into each of the three activation domain vectors (pGAD-C1, pGAD-C2, and pGAD-C3) to generate various fusions for each translational reading frame. Each ligation reaction was transformed into electrocompetent *E. coli* DH10B [F¯*mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) ϕ80*lac*ZΔM15 Δ*lac*X74 *rec*A1 *end*A1 *ara*D139 Δ(*ara*, *leu*)7697 *gal*U *gal*K λ-*rps*L(Str^R) *nup*G; Invitrogen Life Technologies] to recover the library plasmids. To generate genomic libraries based on reading frame, cultures representing a given pGAD vector were combined proportionally, based on the expected restriction frequency for each enzyme. Twenty-five to thirty-five milligrams of each *Y. pestis* library pYP-C1, pYP-C2, and pYP-C3 were purified by large-scale plasmid DNA purification (Qiagen QIAfilter Plasmid Giga Kit).

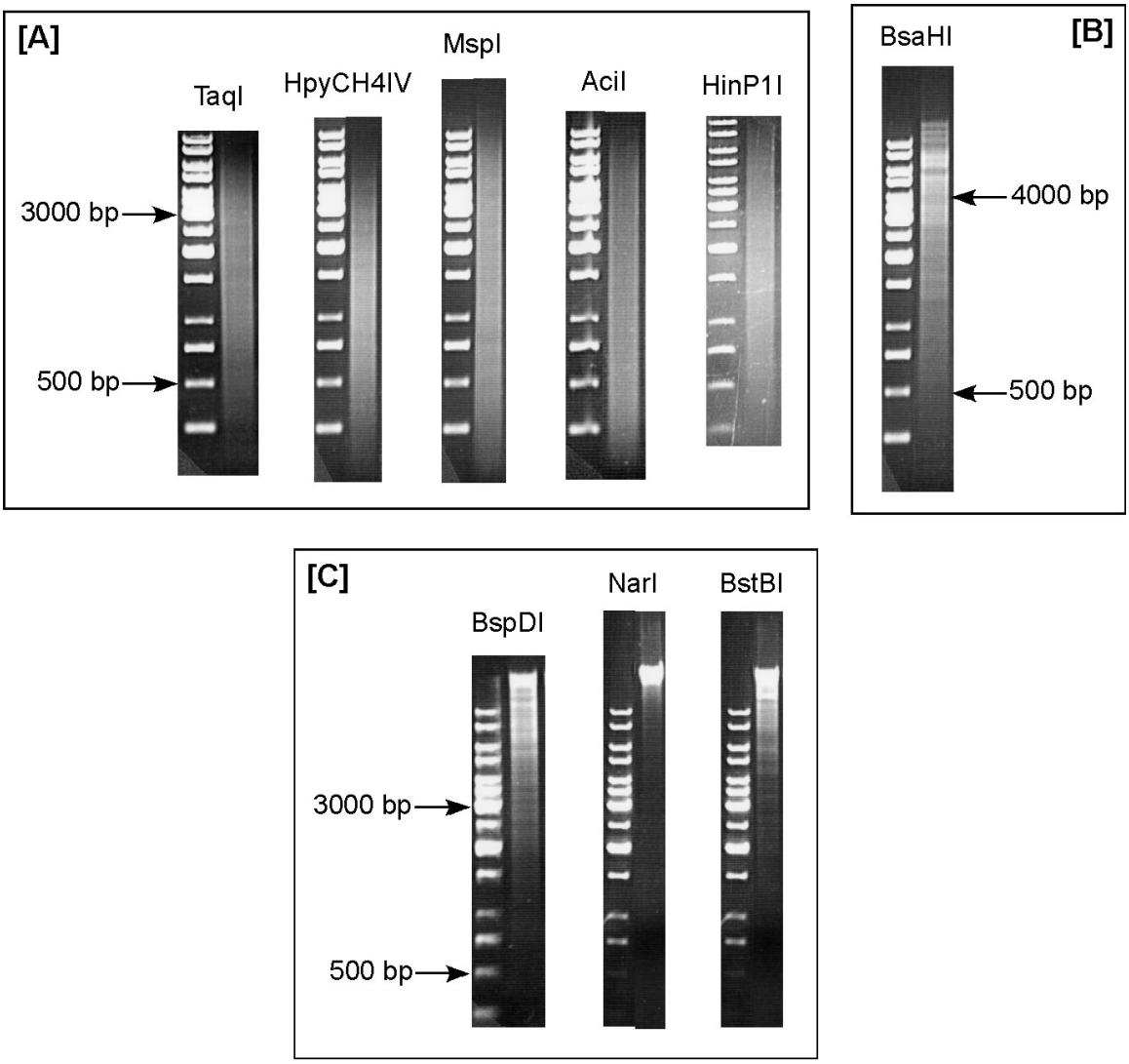

Fig. 5.2. Restriction data for restriction endonucleases (REases) considered for *Y. pestis* yeast two-hybrid library preparation. Digested DNA obtained from reactions using various enzyme to DNA ratios were analyzed by agarose gel (0.5%) electrophoresis. [A] Five REases (TaqI, HpyCH4IV, MspI, AciI, HinP1I) cleaved *Y. pestis* genomic DNA at high frequencies. 500 bp to 3000 bp genomic DNA fragments were purified from these digests and cloned into the pGAD-C1, pGAD-C2, and pGAD-C3 vectors. [B] BsaHI cleaved *Y. pestis* genomic DNA with a lower frequency. 500 bp to 4000 bp genomic DNA fragments were purified from this digest for cloning into the pGAD vectors. [C] Three REases (BspDI, NarI, BstBI) were unable to cleave *Y. pestis* genomic DNA at high frequency and were not used to prepare genomic DNA inserts.

To check the quality of my *Y. pestis* activation domain fusion libraries, I analyzed the inserts from numerous library plasmids. Forty-eight individual plasmids were sampled from each library for this purpose. REases SmaI and PstI cleave at unique sites flanking the ClaI insertion site in the pGAD vectors and were used to release library plasmid inserts for electrophoretic analysis. While plasmids carrying inserts with SmaI and/or PstI restriction sites were expected to produce multiple insert fragments during REase treatment, most reactions were observed to release intact inserts (Fig. 5.3). In each case where multiple fragments were generated, the sizes of these fragments were added to determine the actual insert size. I found that 99% of the plasmids I sampled contained insert and 90% of the inserts I obtained were within my desired insert size range of 500 bp to 3000 bp (Fig. 5.3).

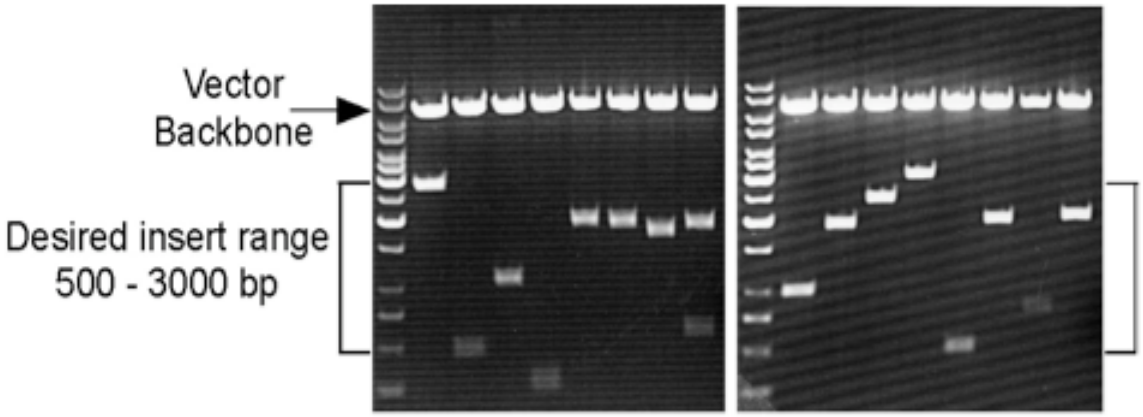

Fig. 5.3. *Y. pestis* yeast two-hybrid library quality assessment. Individual plasmids were isolated from the *Y. pestis* activation domain fusion libraries using the QIAprep Spin Miniprep Kit. Plasmids were subjected to digestion by the endonucleases SmaI and PstI to release plasmid inserts. Digest reactions were analyzed by agarose gel (1.0%) electrophoresis to determine the presence of insert and to estimate insert sizes. 99% of the plasmids sampled contained insert and 90% of the inserts were within the desired insert size range. Representative assay results are shown.

Former Karzai laboratory member Dr. Nihal Okan had generated two bait plasmids using the LexA DNA binding domain vector pSTT91 [pBTM116 (12) with the *Saccharomyces cerevisiae ADE2* gene] and *Y. pestis smpB* alone or *Y. pestis smpB* and *ssrA* together. Plasmid pLexA-SmpB was designed to express a LexA-SmpB fusion protein and plasmid pLexA-SmpB/SsrA was designed to express a LexA-SmpB fusion and tmRNA. Expression of LexA-SmpB and tmRNA from the appropriate bait plasmids in *S. cerevisiae* should be confirmed. tmRNA that is expressed in *S. cerevisiae* should also be analyzed for proper processing to the mature RNA form.

The bait plasmids constructed by Dr. Okan can be screened against the set of libraries that I have constructed to study the protein-protein interactions that are important for *trans*-translation in *Y. pestis*. The *S. cerevisiae* strain L40 [*Mat***a**, *his3*Δ*200, trp1-901, leu2-3,112, ade2, LYS2(lexAop)*$_4$*-HIS3, URA3(lexAop)*$_8$*-lacZ*; (74)] has been successfully used in the past for yeast two-hybrid screening and is a suitable strain for screening with the bait and library plasmids I have described. Positive bait-prey interaction within L40 drives expression of the *HIS3* and *lacZ* reporter genes for straightforward detection of the yeast two-hybrid protein interaction.

Since SmpB and tmRNA mutually depend on each other to function in *trans*-translation and neither of them has been shown to have alternative functions, one would have to be very cautious with data obtained from a screen using the LexA-SmpB alone as bait. There certainly is potential for producing accurate data based on independent SmpB interactions, but candidates should

be rigorously confirmed using binding assays designed to reflect *trans*-translation function. However, the possibility of an interaction representing a novel function of SmpB should also be explored based on the identity of the candidate. The results from a library screening using pLexA-SmpB/SsrA should produce data that complement and expand upon the results from a screen using the LexA-SmpB bait.

Targeted Degradation of Transfer-Messenger RNA-Tagged Proteins

From a shortsighted standpoint, the rescue of trapped ribosomes provides immediate and satisfactory relief for the cell, since nonfunctional ribosomes are essentially dead-end energy sinks that are unable to carry out their necessary purpose. The stress of interrupted translation is certainly intensified by the high efficiency of ribosomes under favorable conditions and the ability of ribosomes to cooperate as polysomes. However, evolution has supported the proteolytic function of *trans*-translation, which suggests that the targeted degradation of incomplete or otherwise abnormal proteins is also quite important. While the inhibition of translation represents an urgent problem, the accumulation of abnormal proteins has great potential for causing cellular stress as well. The physiological burden of abnormal proteins is exemplified by dominant-negative mutations that control phenotypes and by misfolded proteins that accumulate as insoluble aggregates.

Through my efforts to characterize Lon protease as a component of *trans*-translation, I have confirmed that it plays an important role in the degradation of tmRNA-tagged proteins. I have shown that cells defective in Lon are unable to efficiently degrade tmRNA-tagged proteins and I clearly determined that Lon preferentially degrades tmRNA-tagged proteins *in vitro*. Importantly, I have described the contribution of Lon to the turnover of tmRNA-tagged proteins *in vivo* in the context of the ClpXP and ClpAP proteases. My results showed that while Lon does not play as great a role in the cellular turnover of tmRNA-tagged proteins as ClpXP, Lon does play a larger role than ClpAP. It seems likely that important information about the process of tmRNA-tagged protein degradation will continue to unfold.

The Lon, ClpXP, ClpAP, FtsH, and Tsp proteases have different specificities and play distinguishable roles in bacteria (58), yet have all been shown to participate in the degradation of tmRNA-tagged proteins (34, 60, 72, 98). Furthermore, the ClpXP and ClpAP proteases have adaptor proteins that influence their activity against tmRNA-tagged proteins in the cell (42, 115). The breadth of proteases and supporting adaptor proteins that participate in the proteolytic function of *trans*-translation suggests that there should be a regulatory framework supporting the targeted degradation of tmRNA-tagged proteins. Protease activity is also influenced by proteins or cellular metabolites that function in pathways that are distinct from *trans*-translation (41, 109, 161). Therefore, there is no reason to believe that the contribution of each protease to the degradation of tmRNA-tagged proteins remains static throughout the growth

cycle of bacteria and during various stress responses. Experiments designed for the analysis of tmRNA-tagged protein turnover in various protease mutant strains during different states of growth or cell stress should provide important information about relative protease contribution. Studies carried out with mutants that are defective in more than one protease, however, should be undertaken with caution, as the removal of multiple proteases that have both specific and general cellular functions (59) may introduce variables that complicate data interpretation. This drawback was indeed encountered in my attempts to study tmRNA-tagged protein degradation in mutants with multiple protease defects (data not shown).

Further *in vitro* studies using Lon protease against tmRNA-tagged substrates should directly address the kinetics of Lon degradation of tmRNA-tagged proteins. In my *in vitro* proteolysis experiments, I observed that Lon degraded tmRNA-tagged λ-CI-N protein more efficiently than GFP-ssrA. This finding suggested that the nature of the protein substrate modified with the tmRNA tag influences Lon activity, in contrast to what has been observed for the Clp proteases (60, 104, 164). It is also possible that Lon is more sensitive to differences in tmRNA tag presentation. Experiments with the tmRNA tag placed in alternative locations relative to the substrate protein may aid in characterizing Lon activity as well, although such constructs would not be accurate representations of cellular tmRNA-tagged proteins.

The specific Lon recognition determinants harbored within the tmRNA tag is also an important topic for consideration. An analysis of ClpX, ClpA, and SspB

determinants within the tmRNA tag revealed specific tag residues that are critical for proper recognition by each protein (52). Importantly, the ClpX adaptor SspB binds the tmRNA tag in regions that preclude recognition by ClpA but not ClpX, thus illustrating how an adaptor might sequester ClpXP substrates. Since the recognition determinants for ClpX and ClpA are concentrated at the C-terminus of the tmRNA tag, proteins modified by tmRNA tag variants such as tmRNA^{H6} and tmRNA^{DD} are considerably resistant to degradation by Clp proteases (60). However, my observation that tmRNA^{H6}-tagged proteins accumulate in *lon* mutant cells suggests that Lon is active against proteins carrying the tmRNA^{H6} tag. Consistent with this possibility, I observed that *clp* mutants accumulate only wild-type levels of tmRNA^{H6}-tagged proteins in my endogenous protein tagging assays. The location of Lon determinants at the N-terminus of the tmRNA peptide tag is a compelling and reasonable possibility, considering that the C-terminus of the tag is already rich in Clp determinants. Comparative experiments performed with tmRNA^{H6} N-terminal tag variants should help address the question of Lon recognition determinants within the tmRNA tag.

Proteolysis experiments may also be designed based on bacterial species other than *E. coli*. The expansive amount of available genomic information aids in the investigation of species that are not considered model organisms and, thus, are less well studied and understood. It is apparent that while both the Clp and Lon proteases are well conserved throughout bacteria, neither group is thoroughly conserved and some species contain homologs of one group but not of the other (29, 58, 194). However, *ssrA* and *smpB* are completely conserved

throughout bacteria. Therefore, the incomplete conservation of proteases supports the possibility of overlapping protease specificities or an inherent suitability of proteases for specificity modification during the course of evolution. Since ClpXP appears to be the dominant protease for the turnover of tmRNA-tagged proteins in *E. coli*, which expresses both Clp and Lon proteases, it would be very interesting to study the nature of tmRNA-tagged protein degradation in bacterial species that lack Clp, such as several *Mycoplasma* species.

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