Functional characterization of ribosomal protein L11 mutants in Escherichia coli

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FUNCTIONAL CHARACTERIZATION OF RIBOSOMAL PROTEIN L11 MUTANTS IN *ESCHERICHIA COLI*

By

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Master of Science

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Ribosomes carry out the production of proteins for all living organisms. The *Escherichia coli* ribosome consists of three molecules of RNA, and 55 proteins which are structurally and functionally significant. This study centers around the L11 protein, located on the large subunit of the ribosome and part of the GTPase center. The GTPase center interacts with factors necessary for translation that use GTP hydrolysis to provide energy for their reaction; these translation factors are IF-2, EF-G, EF-Tu, RF-3 and RRF. Furthermore, the GTPase center is a known antibiotic binding region for thiostrepton, with resistance arising to this antibiotic through mutations in the L11 protein among others. This study characterizes *E. coli* mutants lacking all or part of the L11 protein through *in vitro* translation experiments and examines the effects of three antibiotics: thiostrepton, spectinomycin, and streptomycin. The study also characterizes the cell lysate, or s100 fraction, from the L11 minus strain and attempts to elucidate the cause of its apparent stimulation of *in vitro* translation.
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Chapter 1

Introduction

The ribosome is a subcellular particle comprised of ribonucleic acid (RNA) and proteins. All living organisms depend on its function for protein synthesis. The *Escherichia coli* ribosome consists of a large (50S) and a small (30S) subunit, each composed of RNA and protein. Three molecules make up the RNA component: the 23S ribosomal RNA (rRNA) and the 5S rRNA on the large subunit, and the 16S rRNA on the small subunit. There are approximately 34 proteins associated with the large subunit, and 21 proteins with the small subunit. The presence of these proteins is essential; however the precise individual function of every protein is not known. The research described in this thesis examines the L11 protein on the large subunit of the ribosome. It has been suggested that L11 is necessary for enhancing the binding and functionality of the GTPase translation factors and it interacts directly or indirectly with several ribosome binding antibiotics. This study provides a functional characterization of a series of *E. coli* mutants that differ in the L11 protein, examining the effects on *in vitro* translation in the presence and absence of antibiotics.

Central Dogma of Molecular Biology

The genetic information for all organisms is stored in their DNA genome. This genetic material contains all of the information necessary to construct a fully functional organism. However, the information is not useful unless it is converted to a functional
medium. Ultimately, proteins must be produced to make use of the information encoded within the genome.

In 1958 Crick proposed the central dogma, describing a flow of information in which the DNA of an organism is transcribed to messenger RNA (mRNA) (Crick, 1958). He saw the mRNA as a short lived messenger whose purpose was to relay the message contained within specific stretches, or genes, of the DNA. The amount of mRNA transcribed is often intricately controlled and provides a mechanism of gene regulation. Ribosomes process the mRNA in groups of three nucleotides. For each three nucleotides, or codon, the ribosome assigns one amino acid. As the mRNA passes through the ribosome, peptide bonds are formed between the amino terminus of one amino acid and the carboxyl terminus of another until the entire protein is synthesized.

![Diagram of transcription and translation]

Figure 1.1: The Central Dogma of Molecular Biology

Proteins have many functions and carry out most processes of the cell. They provide the catalytic activity for the metabolic processes of the organism. They also serve as the structural components to cells and function in the transportation mechanisms. Proteins act as communication liaisons between cells, and exported proteins carry out extracellular functions. In essence, the information needed to control all of an organism’s systems is contained in the nucleic acid genome of the cell. The ribosome serves as a
gatekeeper for this information, translating a transcript of the genomic material into functional proteins.

Translation

Ribosomes translate mRNAs in a highly accurate manner to ensure the synthesis of functional proteins. There are at least three adjacent tRNA binding sites in the ribosome where protein synthesis occurs (Rheinberger, Sternbach et al. 1981). It has been well documented that the mRNA progresses from the A (aminoacyl) site to the P (peptidyl) site to the E (exit) site. The mechanism of translation occurs in three distinct steps: initiation, elongation and termination. Each of these steps requires the interaction of the ribosome with additional factors (proteins) for catalysis. An overview of prokaryotic translation follows.

Initiation

Within cells are free ribosomes, 70S complexes, made up of the large and small subunits. Initiation Factor 1 (IF1) and Initiation Factor 3 (IF3) are involved in dissociating the 70S complex into the two individual subunits (Subramanian and Davis 1970; Sobura, Chowdhury et al. 1977). It is has been proposed that IF1 sits in the A site of the ribosome to stabilize the initiation complex and that IF3 separates the subunits so that they are able to bind the mRNA (Dahlquist and Puglisi 2000; Carter, Clemons et al. 2001). The ribosome also must associate with the mRNA. This occurs with the Shine-Dalgarno—Anti-Shine-Dalgarno interaction (Shine and Dalgarno 1974; Sundari, Stringer et al. 1976). The ribosome contains a conserved pyrimidine rich sequence that is
complementary to a purine rich region of the mRNA upstream of the start codon. During this time, IF1 and IF3 stay bound to the 30S subunit. Concurrently, Initiation Factor 2 (IF2) is responsible for aiding in the binding of aminoacylated tRNA charged with formylated methionine (tRNA$^{f_{\text{met}}}$). IF2 is responsible for bringing the tRNA that recognizes the start codon (AUG) of an mRNA to the 30S complex (Sundari, Stringer et al. 1976; van der Hofstad, Foekens et al. 1977).

IF2, like several other translation factors, is a GTPase. GTPases comprise a family of proteins that hydrolyze GTP as an integral role in their reactions. Several translation factors are GTPases and their interactions with the ribosome and ribosomal proteins will be discussed further below.

The IF2-GTP-tRNA$^{f_{\text{met}}}$ complex aligns with the start codon of the mRNA, most commonly AUG. The 30S initiation complex is complete after the three initiation factors and the mRNA are bound. This complex has a high affinity for binding free 50S subunits (Gualerzi, 2000). After the 50S subunit is bound IF3 dissociates from the complex. Upon IF-2 dependent GTP hydrolysis, IF2, GDP, P$_i$, and IF1 are released from the complex. Therefore at the end of the initiation stage, the mRNA is tethered through the 70S ribosome complex. A tRNA charged with fmet sits in the P site, and the A site is empty, waiting for the next aminoacylated tRNA. Initiation is depicted figure 1.2.
Figure 1.2: Translation Initiation
Initiation is depicted above showing the formation of the ribosomal initiation complex with the aid of Initiation Factors 1, 2, and 3 (IF-1, IF-2, IF-3). See text for details. Figure from Matthews, Van Holde, and Ahern, 2000.
Elongation

After initiation, the ribosome's empty A site beckons a cognate charged tRNA (amino-acylated tRNA, aa-tRNA). Elongation Factor Tu (EF-Tu) forms a ternary complex with GTP and aa-tRNA that has a high affinity to the A site of the ribosome. EF-Tu is a GTPase, and it must have a bound GTP for it to bind the tRNA. Upon tRNA binding to the A site, through the codon-anticodon interaction, the GTP is hydrolyzed, and EF-Tu leaves.

As the aa-tRNA is brought to the ribosome by EF-Tu, the ribosome checks for the correct fit and complementarity between the codon and anticodon. This process is necessary to maintain the fidelity of translation, and is accomplished by the 30S subunit. Elongation has been described kinetically as consisting of seven steps, of which several contribute to translational fidelity (Pape, Wintermeyer et al. 1999). The first step is the initial binding of the aa-tRNA—EF-Tu—GTP complex to the A site of the ribosomes. This step is codon independent, and is the first interaction of the codon and anticodon. The second step is the codon recognition. This step involves the interaction of the anticodon of the tRNA and the exposed codon of the mRNA in the A site. The rate constant for this step varies greatly depending on whether or not there is a cognate tRNA bound in the A site. The implications will be discussed later. The third step of elongation is the GTPase activation. Evidence suggests a conformational change in the GTPase center of the ribosome that activates GTPases, including EF-Tu. The fourth step is GTP hydrolysis. GTP hydrolysis occurs upon GTPase activation. The fifth step is the conformation change of EF-Tu. GTP hydrolysis to GDP results in a conformational change of EF-Tu, which changes to accommodate the GDP bound form. This complex
does not have a high affinity for the aa-tRNA, and dissociates from the tRNA ribosomal complex. The sixth step is the accommodation of the aa-tRNA into the A site of ribosome immediately preceding peptidyltransferase. Following EF-Tu dissociation, the aminoacyl end of the tRNA moves into the 50S subunit A site. The seventh step is peptide bond formation. The peptidyltransferase activity of the ribosome appears to occur immediately upon accommodation (sixth step) of the aa-tRNA. These steps are illustrated in Figure 1.3.

Figure 1.3: Kinetics Model of Translation (Pape, Wintermeyer et al. 1999). The model depicted separates the steps of Elongation to obtain kinetics data.

Rate constants of near-cognate aa-tRNA binding and cognate aa-tRNA binding were obtained and analyzed. The study provided evidence that the defining step as to whether the elongation step will proceed is based on the GTPase activation and hydrolysis step. These steps were combined due to the extremely fast nature of
hydrolysis. The GTPase activation was rate limiting according to the work. The kinetics of cognate aa-tRNA binding was 10 times faster than near-cognate binding. Therefore, the ribosome appears to stall in the presence of anything other than cognate aa-tRNAs, allowing them to leave the A site and be replaced by the correct aa-tRNA.

This hypothesis was strengthened by solved crystal structures showing the differences in cognate aa-tRNA binding versus near-cognate aa-tRNA binding. Cognate aa-tRNA binding induced global domain movements in the 30S subunit as well as in the universally conserved and essential bases A1492, A1493, and G530 (Ogle, Brodersen et al. 2001). They showed that bases A1492 and A1493 “flip out” of helix 44 during a conformational change upon recognition of the cognate aa-tRNA. Also, G530 has been shown to switch from the syn conformation to an anti conformation in footprinting assays. These rRNA nucleotides interact with the first two base pairs of the codon-anticodon helix in the A site, which determine much of the specificity. The third position has been labeled the “wobble” position, because of its apparent ability to accommodate base pairs other than the traditional and canonical Watson-Crick base pairs (Crick 1966). The action of bases A1492 and A1493 flipping out of helix 44 gives these bases the ability to directly monitor the codon-anticodon interaction, to ensure translation fidelity.

Once the EF-Tu—GDP complex leaves the ribosome, an additional elongation factor, EF-Ts binds the EF-Tu—GDP complex, and displaces the GDP (Blumenthal, Douglass et al. 1977). Eventually a GTP will displace the EF-Ts from the EF-Tu—Ef-Ts complex providing a EF-Tu—GTP complex ready to bring another tRNA to the ribosome.
Upon recognition of the cognate aa-tRNA, the peptide bond must be formed between the nascent peptide and new amino acid. The peptidyltransferase region of the ribosome, made up of catalytic ribosomal RNA (rRNA), accomplishes this peptide bond formation between the carboxyl group of the amino acid in the P site and the amino group of the amino acid in the A site. This region of ribosomes that accomplishes the peptide transfer appears to be made up entirely of rRNA. According to recent crystal structures, the nearest protein residues were at least 18 Å away (Nissen, Hansen et al. 2000). The peptide bond is formed immediately upon recognition of the cognate tRNA in the A site. At the first round of elongation, it will be between the N-formylmethionine and the amino acid attached to the tRNA in the A site.

The event of translocation also must occur to advance the elongating peptide chain, where the ribosome advances one codon on the mRNA. The tRNA in the A site moves to the P site, and the tRNA in the P site moves to the E site, the exit site for tRNAs in the ribosome. This movement is accomplished with Elongation Factor G (EF-G) which is also a GTPase. When it is determined that the correct tRNA is in the A site of the ribosome, GTPase activation occurs. The GTP is hydrolyzed, and then the EF-Tu—GDP complex leaves the ribosomal complex. Upon accommodation of the aa-tRNA in the 50S subunit, peptidyltransferase spontaneously occurs. At this point, EF-G binds the ribosomal complex, and it moves the tRNA one codon upon its GTP hydrolysis. Lastly, the deacylated tRNA, which was moved to the E site during translocation, is released from the ribosome. The end of the cycle results in the P site occupied with the tRNA that is attached to the growing peptide, and the A site, exposing the subsequent mRNA codon, is ready for the next aa-tRNA. Elongation is depicted in Figure 1.4.
Figure 1.4: Translation Elongation

Elongation is depicted in the figure showing the three binding sites for tRNA and peptide synthesis. Elongation Factors Tu and G assist in the process. Figure from Matthews, Van Holde, and Ahern, 2000.
A widely accepted view of translocation is the hybrid states model, where the A, P and E sites morph into the A/P site and the P/E site as depicted in Figure 1.5 (Moazed and Noller 1989). Instead of the entire tRNA moving discretely through three distinct sites on the ribosome, the extremely fast nature of the peptidyltransferase activity prompted a different hypothesis. This model is based on the anticodon and acceptor ends of the tRNA not moving through the ribosome together. The Noller group examined the tRNA binding sites distinguishing their interactions between the 30S and 50S subunits. The 30S accommodates the anticodon end of the tRNA, whereas the 50S contains the peptidyltransferase region and interacts with the acceptor end of the tRNA. At the beginning of the elongation cycle, the A site is empty and the tRNA with nascent peptide is in the P/P site (the P site of the 30S and the P site of the 50S). As the EF-Tu ternary complex brings the aa-tRNA to the A site, the interaction of the codon and anticodon only occurs in the A site of the 30S subunit. At this stage of elongation, the hybrid-states model termed the new tRNA to be in the A/T site. Upon cognate binding and GTP hydrolysis, the acceptor end of the tRNA moves into the A site of the 50S. The new aa-tRNA, at this step, would have its anticodon in the A site of the 30S and its acceptor end in the A site of the 50S (the A/A site). The tRNA with the nascent peptide would still be in the P/P site. Upon peptidyltransferase, the acceptor end of the new tRNA would move into P site of the 50S. Its anticodon end would still be in the A site of the 30S, therefore its hybrid state is termed the A/P site. Similarly, the tRNA from which the peptide was transferred from would move into the P/E site. After EF-G dependent translocation occurs, the tRNA that was in the P/E site would move into the E site, on the 50 subunit. The tRNA that was in the A/P site would move into the P/P site. The hybrid-states model...
provides a more fluid understanding of how tRNAs move through the ribosome (Figure 1.5).

Figure 1.5: Hybrid States Model (Wilson and Noller 1998)
The figure depicts a model for tRNA movement through the binding sites of the 30S and 50S subunits. The model suggests that the acceptor and anticodon ends of tRNA are not always in the same binding site on both subunits.

An alternative to the hybrid states model was proposed by the Nierhaus group called the alpha-epsilon model. Instead of the three tRNAs moving through the ribosome, the alpha-epsilon model described three tightly bound tRNAs bound to a movable domain within the ribosome. The alpha-epsilon domain is proposed to move the tRNA-mRNA complex from the A and P sites to the P and E sites during translocation and maintains the binding of both tRNAs. (Nierhaus, Stuhrmann et al. 1998). While the hybrid states model adequately explains most of the biochemical structure data for tRNA binding, tRNAs in the hybrid states have not been observed by cryo-EM or
crystallography. However, there are some cryo-EM data that support the alpha-epsilon model (Agrawal, Spahn et al. 2000).

Termination

The ribosome-mRNA complex must have a system of determining when the elongating peptide is complete. The last three nucleic acids of the translatable region of the mRNA make up the stop codon, either UAA, UAG or UGA. Instead of tRNAs binding like a normal stage of elongation, either Release Factor 1 (RF-1) or Release Factor 2 (RF-2) binds. RF-1 recognizes the stop codons UAA and UAG, and RF-2 recognizes UAA and UGA. The third release factor (RF-3) binds to a different region of the ribosome, and is a GTPase (Grentzmann and Kelly 1997).

As the peptidyltransferase transfers the completed peptide, it is released from the tRNA and transferred to a water molecule. The GTP hydrolysis of RF-3 appears to further stimulate the release of the growing peptide (Grentzmann, Kelly et al. 1998). After the peptide is released, all of the RFs leave the ribosome. The end result is the unstable 70S ribosomal complex tethered with an mRNA and bound to a deacylated tRNA. This is depicted in Figure 1.6.

The very last stage of translation (not pictured in figure) could be termed the recycling of the ribosome. At this stage, Ribosome Recycling Factor (RRF), a GTPase and a tRNA mimic, binds the unstable complex. The precise mechanism for this step is not known; however, the 50S subunit dissociates, the deacylated tRNA leaves, and the 30S is ready to be initiated for a new round of translation. RRF is a GTPase that has several functions during translation. It releases ribosomes from mRNA and at the natural
termination codon. RRF is dependent on GTP and either EF-G or RF-3, both of which are GTPases. RRF participates during elongation to reduce errors, and possibly might work in conjunction with EF-G (Kaji and Hirokawa, 2000, and references cited therein). RRF also has a stimulatory effect on protein synthesis (Ryoji, Karpen et al. 1981).
Ribosomal GTPase Center

As discussed in the steps of translation, many of the factors involved are GTPases, which hydrolyze GTP to GDP to provide the energy for their reaction. It was hypothesized as early as 1971 that a single region of the ribosome interacted with all of the GTPase factors. This proposal arose from data that both thiostrepton and siomycin were able to inhibit the function of more that one elongation factor, EF-Tu and EF-G, both of which are GTPases (Cundliffe 1971; Modolell, Cabrera et al. 1971).

Low resolution cryo-electron microscopy (cryo-EM) structures, as well as biochemical studies have shown that the factors interact with several ribosomal proteins, specifically the L10, L11 and the L7/L12 complex (Wriggers, Agrawal et al. 2000; Valle, Sengupta et al. 2002). The L7/L12 complex is made up of a dimer of L7/L12 heterodimers, where the L7 differs from the L12 only by an acetylated N terminus. Previous studies by Savelsbergh, et al. examined the importance of the L7/L12 for the GTPase activation of Elongation Factors Tu and G (Savelsbergh, Mohr et al. 2000). They reported that the EF-G GTP hydrolysis was stimulated by the isolated L7/L12 complex, where as EF-Tu GTP hydrolysis was not. However, the rate of GTP hydrolysis stimulated by the isolated L7/L12 was still about 500-fold less than that of the entire ribosome. The mechanism of GTPase activation is still not elucidated; however, advances have been made. Cryo-EM maps in combination with molecular modeling have shown interactions between GTPases and the GTPase center on the ribosome. It
was hypothesized that an arginine finger mechanism may be involved because of a conserved arginine residue in the factors EF-Tu and EF-G; however replacement of the residue did not significantly affect GTPase activity (Zeidler, Egle et al. 1995). Domain V of EF-G was shown to extend down into the cleft between 23S rRNA and the N-terminal domain of L11 (Agrawal, Linde et al. 2001). Recent work has suggested the α-sarcin loop in the 23S rRNA extends over into the binding region and is involved in activating GTPases (Stark, Rodnina et al. 2002). It has also been suggested that not all GTPase factors interact with the region in an identical manner. The hypothesis suggesting the synergistic nature of EF-Tu and EF-G describes how each factor has different affinities to the translation complex based on what conformation it is in (Mesters, Potapov et al. 1994).

L11, like the other proteins in the GTPase center, has been shown to play an essential role in protein synthesis. It was originally thought that L11 made up the GTPase center for the ribosome based on the initial work with thiostrepton and siomycin (Cundliffe 1971; Modolell, Cabrera et al. 1971). The binding site for thiostrepton was determined to include the L11 protein through experiments examining antibiotic sensitivity with and without the protein (Egebjerg, Douthwaite et al. 1989). Ribosomes that were lacking the entire protein were less sensitive to the antibiotic than wild type ribosomes observed in \textit{in vitro} studies.

The L11 protein sits at the base of the L7/L12 stalk region on the 50S subunit of the ribosome. The rRNA binding region of the L11 consists of nucleotides 1051-1108 in \textit{E. coli} (Thompson, Cundliffe et al. 1979; Schmidt, Thompson et al. 1981). The protein is approximately 14.8 kDa and made up of 140 amino acids. L11 is highly conserved
across many organisms suggesting the magnitude of its importance. Crystallographic studies of the ribosome have offered great insight into the structure; however, this particular region is rather flexible, especially the N-terminal domain of L11. Therefore, it cannot be seen in many of the crystallographic structure models.

(Ban, Freeborn, et al. 1998)

Figure 1.7: Location of ribosomal protein L11
The L11 protein is located on the 50S subunit at the base of the L7/L12 protein complex. In the rasmol figure on the right, L11 is shown in red, with the N-terminal domain hanging down.

The importance of the L11 as part of the GTPase center is evident, yet the precise function is still not known. Bacterial strains lacking the entire L11 protein are still viable, which offers us an interesting study system. To better study this protein, we utilize several strains of *E. coli* which lack all or part of the L11 protein. These strains were produced in the Murgola laboratory and are described in a recent publication (Van Dyke, Xu et al. 2002). The wild type strain is labeled NVD001. The mutant strains were made by knocking out the entire gene encoding the L11 protein, and subsequently
supplying an intact or truncated L11 extrachromosomally by transformation with various plasmids. NVD002 harbors the N-terminal deletion of L11, where only the C-terminus has been added back on a plasmid. NVD005 is the L11 minus strain, which is missing the entire protein.

Antibiotics

The L11 protein and the L11 binding region of the rRNA have been shown to interact with several antibiotics, specifically with thiostrepton and micrococcin among others (Cundliffe, Dixon et al. 1979; Spedding and Cundliffe 1984; Porse, Leviev et al. 1998; Porse, Cundliffe et al. 1999). In fact, some of the antibiotic resistant strains have been shown to lack the N-terminus of L11 or the entire protein (Stoffler, Cundliffe, et al. 1980).

Thiostrepton most likely binds the 50S subunit in the cleft between the 1067 and 1095 loops, and the N-terminal domain of the L11 protein. It has been shown to be a translocation inhibitor (Pestka 1970; Hansen, Ippolito et al. 2002). Nearly thirty years ago, L11 was shown to be necessary for the binding of thiostrepton (Highland, Howard et al. 1975; Cundliffe, Dixon et al. 1979). Similar results establishing the necessity of L11 were found with a similar thiazole antibiotic, micrococcin (Spedding and Cundliffe 1984).

Spectinomycin also is known to be a translocation inhibitor; however, it binds the 30S subunit (Kostiashkina, Asatrian et al. 1975; Bilgin, Richter et al. 1990; Ramakrishnan and White 1992; Carter, Clemons et al. 2000). Streptomycin has an interesting relationship to spectinomycin. Previous studies have shown that mutations in
S5 can offer spectinomycin resistance or a reversion from streptomycin dependence (Ramakrishnan and White 1992). Genetic loci for streptomycin resistance and spectinomycin resistance are closely linked on the *E. coli* chromosome, and are considered part of a group of markers that define structural elements for the 30S subunit (Flaks, Leboy et al. 1966). Previous evidence shows that streptomycin causes misreading of the mRNA, or a loss of fidelity that is normally maintained by the 30S subunit (Ramakrishnan and White 1992). Also, GTPase effects have been related to the presence of streptomycin. Previous studies show that streptomycin stimulates ribosome-dependent GTPase activity (Novogrodsky 1971) and peptide synthesis (Tai, Wallace et al. 1978). In thermophilic bacteria, it was observed that the presence of streptomycin stimulated *in vitro* protein synthesis (Uzawa, Yamagishi et al. 2002).

**Stringent Response and LI1**

More recent studies have shown LI1 to be an integral protein in the stringent response. This response was discovered over 30 years ago with the discovery of ppGpp, or magic spot (Cashel 1974; Cashel 1975; O'Farrell 1978). It was realized that ribosomes in starved environments induce response mechanisms. Specifically, if the environment is starved for amino acids, ribosomes are likely to have deacylated tRNAs in the A site. This situation induces the stringent response which is mediated through RelA and SpoT (Raue and Cashel 1975; Stephens, Artz et al. 1975; Seyfzadeh, Keener et al. 1993; Fujita, Nishimura et al. 2002). RelA is responsible for producing ppGpp when the stringent response is activated. The mechanism of ppGpp production is still not elucidated, but some effects are known. In the presence of ppGpp, transcription of many genes is
decreased (Price and Brown 1981) and ribosome biosynthesis is decreased (Reiness, Yang et al. 1975). When it is determined that the environment is back to normal, SpoT is responsible for degrading the ppGpp (Raue and Cashel 1975; Seyfzadeh, Keener et al. 1993). In certain circumstances, it is possible for SpoT to be driven in the reverse direction, producing ppGpp (Fujita, Nishimura et al. 2002).

Recently, it was discovered that L11 is necessary for the production of (p)ppGpp (Wendrich, Blaha et al. 2002). These results suggested that RelA can bind the ribosome in the presence or absence of L11; however, it cannot produce ppGpp unless L11 is present. Another study examined mutants that displayed relaxed phenotypes; they inhibited the accumulation of (p)ppGpp (Yang and Ishiguro 2001). These mutations were mapped to two genes, the relA gene and relC. relC was later discovered to be the same as rplK, the gene that produces the L11 protein. The characterization of these mutants differing in the L11 protein may offer additional insight to the mechanism and function of the stringent response.

Summary

The experiments described in this thesis offer insight into the L11 protein via a functional characterization of a series of L11 mutants. Growth characteristics are described followed by in vitro translation data from all three strains in combination with the cell lysates from all three strains of bacteria. In vitro translation data are presented in the presence of three antibiotics: thiostrepton, spectinomycin, and streptomycin. Thiostrepton is a translocation inhibitor and binds directly in the GTPase center of the ribosome and resistance to this antibiotic has been related to the L11 protein. Translation
data suggest that the wild type strain is most sensitive to the antibiotic and the translation capability is severely limited at stoichiometric concentration of ribosomes and thiostrepton. The N-terminal minus strain ribosomes are less sensitive to the antibiotic, and the L11 minus ribosomes show the least sensitivity to thiostrepton. Spectinomycin is also a translocation inhibitor but binds in the 30S subunit of the ribosome. The three strains of ribosomes were affected similarly by spectinomycin. Streptomycin affects the fidelity of translation, and its binding region is also in the 30S subunit. At low concentration of streptomycin, all ribosomes tested showed similar inhibition of translation. However, higher concentrations of streptomycin appear to partially rescue the translation ability of wild type ribosomes. This rescue mechanism was not evident in the L11 mutant ribosomes.

The third chapter explores an apparent stimulatory effect of NVD005 lysate on *in vitro* translation. Multiple experiments are described to better characterize what is causing the stimulatory effect. These experiments include variations of *in vitro* translation conditions, with kinetics data, poly-U translations, Initiation Factor titrations, and sub/superstoichiometric mRNA experiments. Also the relation of L11 and the stringent response was investigated, specifically examining the production of ppGpp due to RelA and SpoT. The thesis finishes with conclusions considering all of the research presented, and offers perspectives on future possibilities.
Chapter 2

In Manuscript Form to be Submitted for Publication

Functional Characterization of Ribosomal Protein L11 Escherichia coli Mutants in the Presence of Thiostrepton, Spectinomycin, and Streptomycin

Introduction

Ribosomal proteins modulate the structure and function of the ribosome and facilitate interactions with translation factors during several steps of protein biosynthesis. Several of these translation factors are GTPases that interact with one specific region of the ribosome, termed the “GTPase center.” (Cundliffe 1971; Modolell, Cabrer et al. 1971; Cundliffe, Dixon et al. 1979). This region is comprised of ribosomal proteins L10, L11 and the L7/L12 complex, ribosomal RNA (rRNA) in the L11 binding region including nucleotides 1051-1108, and the sarcin-ricin loop of rRNA. Initiation Factor 2 (IF-2), Elongation Factors Tu and G (EF-Tu and EF-G) and Release Factor 3 (RF-3) are all GTPases and interact with the GTPase center. Additional translation factors that are not GTPases, including RF-1 and RF-2, also interact in this region (Wilson, Ito et al. 2000; Van Dyke, Xu et al. 2002).

The highly conserved GTPase center has been suggested to aid in factor binding to the ribosome. Cryo-EM maps with molecular modeling show that following EF-G dependent GTP hydrolysis, domain V of EF-G extends down into the cleft between the 23S rRNA and the N-terminal domain of L11 (Agrawal, Linde et al. 2001). The GTPase center also likely is involved in activating the GTPase factors in some manner; however, the precise mechanism remains unknown. It was suggested that several conserved
arginine residues in the L10, L11, and L7/L12 proteins might be involved in an arginine-finger type mechanism (Zeidler, Egle et al. 1995). More recent work suggests that the α-sarcin loop extends over and is involved in activating the GTPases (Stark, Rodnina et al. 2002). Another study examined the involvement of the L7/L12 complex in the activation of EF-G. It concluded that while the L7/L12 complex does stimulate EF-G dependent GTP hydrolysis, the stimulation is 500-fold less than that of the entire ribosome (Mohr, Wintermeyer et al. 2002). Clearly, more elements are involved in stimulating the activation of the GTPase translation factors.

The N-terminal domain of L11 has not been resolved in the recent crystal structures due to its high level of flexibility. Conformational changes occurring within the GTPase center may be involved in activating the GTPase factors during translation. It has been shown that L11 protein is involved with several events during translation, including translocation and termination (Agrawal, Linde et al. 2001; Van Dyke, Xu et al. 2002). Additional evidence suggests that RF-1 interacts with the L11 protein during termination (Van Dyke, Xu et al. 2002).

The L11 region has been implicated as an antibiotic binding region of the ribosome. Thiostrepton likely binds the 50S subunit in the cleft between the 1067 and 1095 step loops and the N-terminal domain of L11. It has been shown to be a translocation inhibitor (Pestka 1970; Hansen, Ippolito et al. 2002). Nearly thirty years ago, L11 was shown to be necessary for the binding of thiostrepton (Highland, Howard et al. 1975; Cundliffe, Dixon et al. 1979). Similar results establishing the necessity of L11 for antibiotic binding were shown for a similar thiazole antibiotic, micrococcin (Spedding and Cundliffe 1984).
Spectinomycin also is known to be a translocation inhibitor; however, it binds the 30S subunit (Kostiashkina, Asatrian et al. 1975; Spedding and Cundliffe 1984; Ramakrishnan and White 1992; Carter, Clemons et al. 2000). Streptomycin was the third antibiotic used in this study because of its close relation to spectinomycin (Flaks, Leboy et al. 1966). Previous evidence shows that streptomycin causes misreading of the mRNA, or a loss of fidelity that is maintained by the 30S subunit (Old and Gorini 1965; Ramakrishnan and White 1992). Also, GTPase effects have been related to the presence of streptomycin. Previous studies show that streptomycin stimulates ribosome-dependent GTPase activity (Novogrodsky 1971) and peptide synthesis in vitro (Tai, Wallace et al. 1978). In thermophilic bacteria, it was observed that the presence of streptomycin also stimulated in vitro protein synthesis (Uzawa, Yamagishi et al. 2002).

The importance of ribosomal protein L11 in translation and cell function is clear; however, precise mechanisms of its involvement are not known. The viability of mutant bacteria that lack all or part of this protein enables the study of its effects on translation and other affected processes in the cell. This study utilizes three E. coli strains that differ in the L11 protein. In addition to the wild type ribosomes produced by the parental strain, one strain produced ribosomes lacking the N-terminal domain of L11. Another strain produced ribosomes completely lacking ribosomal protein L11 (Van Dyke, Xu et al. 2002).

This study offers insight into the translational characteristics of ribosomal protein L11 mutants in the presence and absence of three different antibiotics. In the presence of thiostrepton, we observed a lower level of translation inhibition with the mutant ribosomes than wild type ribosomes. Inhibition of translation due to the 30S specific
translocation inhibitor, spectinomycin, was similar in all three strains. Finally, low concentrations of streptomycin inhibited wild type and mutant ribosomes. However, increasing concentrations of streptomycin actually improved translation on wild type ribosomes, but not mutant ribosomes. Characterization of these mutants in these environments can offer functional insight into the L11 protein.

Materials and Methods

The strains of *E. coli* were grown to early log phase at 37°C in Luria broth with vigorous agitation. Cells were harvested by filtering the culture through the Millipore Pellicon cassette system. The cells were washed with Nieremberg Buffer, NB (10 mM MgAcetate, 60 mM Ammonium Chloride, 10 mM Tris-Cl, pH=7.7), then pelleted at 10,000 rpm (Sorvall GSA rotor) for 10 minutes and stored at -80°C.

To purify the ribosomes, approximately 17 g of cells were ground with 25.5 g alumina at 4°C for about one hour. This resulted in approximately 25 mL of grindate, to which benzamidine was added to a final concentration of 0.2 mM, PMSF was added to a final concentration of 0.2 mM, and β-mercaptoethanol was added to a final concentration of 6 mM. The grindate was ground again for approximately 5 minutes and then 25 mL of NB were added, along with benzamidine, PMSF, and β-mercaptoethanol to maintain their concentrations. The grindate was centrifuged at 12,000 rpm for 10 minutes at 4°C (SS34 rotor) to pellet the cell debris and alumina. The supernatant was separated and spun at 40,000 rpm (Ti70 rotor) for 6 hours at 4°C to pellet the ribosomes. The supernatant resulting from this spin is the s100 fraction. The s100 was dialyzed twice for two hours against 1X NB, 10%glycerol, 0.2 mM benzamidine, 0.2 mM PMSF, and 6 mM β-
mercaptoethanol. After dialysis, the s100 was aliquotted and stored at -80°C. Ribosomes were washed three times with 15 mL ribosome washing buffer, RWB (100 mM Tris-Cl, 10 mM Magnesium Acetat, 600 mM NH₄Cl, 50 mM KCl, pH=7.7), and resuspended in 25mL of RWB with 2mM β-mercaptoethanol. The resuspended ribosomes were spun again at 28,000 rpm for 6 hours at 4°C (Ti70 rotor) to separate out more cell debris. The supernatants were transferred to sterile tubes and spun at 60,000 rpm for 2.5 hours at 4°C to pellet the ribosomes (Ti70). The pelleted ribosomes were washed three times with RWB (2 mM β-mercaptoethanol) and resuspended in 2 mL 1X NB with 2 mM β-mercaptoethanol. The purified, rough 70S ribosomes were aliquotted and stored at -80°C.

In Vitro Translation

Translation was measured through an in vitro system that assays for the amount of radiolabeled peptide synthesized. Purified, salt-washed ribosomes were combined with exogenous initiation factors 2 and 3 to a final concentration of 3 μM for each in Nieremberg Buffer, NB, (10 mM Magnesium Acetate, 60 mM NH₄Cl, 10 mM Tris-Cl, pH=7.7). IF-1 does not affect this in vitro translation system (data not shown). The additional translation factors come from the cell lysate, specifically the supernatant after centrifugation at 40,000 rpm for 6 hours during the ribosome purification process. This fraction contains proteins in the cell at the time of lysis, including the elongation, termination and other translation factors necessary for in vitro translation.

An additional mixture was made containing the amino acids necessary for translation, along with the following components: ATP (5 mM), GTP (5 mM),
Dithiothreitol (5 mM), Phosphoenol Pyruvate (25 mM), Pyruvate Kinase (1.25 mM), 5-10-Methenyltetrahydrofolate, MTHF (333 μM), ^14C-Phenylalanine (25 μM), phenylalanine (100 μM), amino acids dependent on the messenger RNA (500 μM each), bulk tRNA (300 μM), messenger RNA (2.75 μM), 10X NB, and nanopure H2O. MTHF was prepared and donates the formyl group to methionine to produce met-tRNA^fmet for the initiation of translation. The mRNA used in the experiments encoded 19 amino acids, including six phenylalanines, a methionine, threonines and isoleucines. It contained both start and stop codons, to function similar to a natural messenger RNA. The preparation and composition are described later in detail. All other components were purchased from Sigma.

Eight μL of the ribosome-IF mixture were added for each reaction, along with 3 μL of s100 and 12 μL of the amino acid mixture. NB was added to bring the reaction volume to 30 μL. Two μL of antibiotics were added to the reaction at this time when noted. The concentration of antibiotic was achieved by adding the same volume from different stock concentrations to ensure the same amount of solvent was added to each reaction. The tube was incubated for the desired time at 37°C. After incubation, 20 μL of each reaction were spotted onto filter paper and dropped into a beaker with a 10% Trichloroacetic Acid solution (TCA) and placed on ice for at least 30 minutes. The filters were transferred to a beaker containing 5% TCA and that vessel was placed in boiling water for 10 minutes. Next, the filters were washed three times with fresh 5% TCA at room temperature, again with a 1:1 Ether:Ethanol solution, and finally with pure Ether. The filter paper was allowed to dry at room temperature and placed in scintillation tubes.
ScintiSafe 30% scintillation liquid was added and the amount of radiolabeled peptide was counted in a Packard Liquid Scintillation Analyzer.

mRNA Preparation

The mRNA was prepared by ligating together oligonucleotides to form the following DNA template, that is preceded by the T7 promoter:

5' TACT ATG TTT ACG ATT ACT ACG ATC TTC TTC ACT TTT ACG ATT 3'
ATGA TAC AAA TGC TAA TGA TGC TAG AAG AAG TGA AAA TGC TAA
.ACT ACG ATC TTC TTC ACT TAA 3'
TGC TGC TAG AAG AAG TGA ATT 5'

The sense oligonucleotide contained the T7 promoter. The sense oligonucleotide, the 3'-antisense oligonucleotide, and the 5'-antisense oligonucleotide were incubated with T4 ligase and ligase buffer (50 mM Tris-Cl, 10 mM MgCl$_2$, 10 mM DTT, 1mM ATP, 25 μg/ml BSA, pH=7.5) for 2 hours at 25°C to form the DNA template. This template was gel purified and then transcribed using an Epicentre T7 Transcription Kit. The result was the following RNA:

5' UACU AUG UUU ACG AUU ACU ACG AUC UUC UUC ACU UUU
ACG AUU ACU ACG AUC UUC UUC ACU UAA AAG 3'

This messenger RNA would lead to the following peptide upon translation as described above:

$^{13}$NH$_3$-met-phe-thr-ile-thr-thr-ile-phe-phe-thr-thr-ile-thr-thr-ile-phe-phe-thr-COO$^-$
Results

The various strains of bacteria grew at different rates depending on the mutations present. Absorbances were taken at 600 nm during growth to measure doubling times. The absorbances are plotted in Figure 2.1.

![Growth Curves of isogenic E. coli strains differing in the L11 protein. The wild type is labeled NVD001, L11 N-terminal minus is NVD002, and L11 minus is NVD005. Curves were fitted to the data to obtain the equations shown.](image)

Figure 2.1: Growth Curves of isogenic E. coli strains differing in the L11 protein. The wild type is labeled NVD001, L11 N-terminal minus is NVD002, and L11 minus is NVD005. Curves were fitted to the data to obtain the equations shown.

Growth rates are similar to those reported in previous studies of L11 deficient strains. The doubling time for the wild type strain was 40 minutes. The NVD002 strain, N-terminal minus, grew about half as fast as the wild type strain. Its doubling time was approximately 72 minutes. The L11 minus strain was affected most. It grew about six times slower than the wild type, with a doubling time of 240 minutes. These characteristics are similar to previous studies; the N-terminal minus strain at about half
the rate of the wild type strain and the L11 minus strain grew approximately six to eight times as slow.

*In vitro* translation was used to further characterize the effects of the different mutations. Translation was conducted with the 022 synthetic messenger RNA described in Materials and Methods, encoding six phenylalanines. All combinations of ribosomes and s100 fractions were tested. The results are seen in Figure 2.2. The wild type ribosomes were able to translate the mRNA to a much greater extent than either N-terminal minus strain or the L11 minus strain in 30 minute translation assays.

![Translation of NVD Ribosomes](image)

**Figure 2.2:** Translation of NVD Ribosomes in combination with all s100 Fractions. Translation was stopped after 30 minutes with all combination of ribosomes and s100. NVD001 s100 is shown in blue, NVD002 s100 is shown in maroon, and NVD005 s100 is shown in yellow. 022 mRNA was used. Error bars represent the standard deviation.

**Antibiotic Binding**

The effect of antibiotics on the various strains of bacteria was measured using the *in vitro* translation with increasing concentrations of thioestrepton, spectinomycin, or...
streptomycin. NVD005 s100 was used with all three strains of ribosomes. This s100 was
chosen because it does not contain any of the L11 protein. It is possible that the other
cell lysates contain fragments of the L11 or the entire protein and could compromise the
L11 status of the mutant ribosomes. The ribosomes were allowed to translate for 30
minutes.

Thiostrepton

The first antibiotic used was thiostrepton. This antibiotic binds directly in the
GTPase region of the ribosome interacting with nucleotides A1067-A1098 (Egebjerg,
Douthwaite et al. 1989). Antibiotic ranged from 0.1 μM to 10 μM and the results can be
seen in Figure 2.3. Due to the chemical nature of thiostrepton, it was dissolved in
dimethyl sulfoxide (DMSO) instead of water. Small concentrations of DMSO stimulate
translation by considerable amounts (up to 40%, data not shown), therefore the results
need to be interpreted accordingly. To help control for the stimulation, each translation
mixture contained the same final concentration of DMSO.

The wild type ribosomes were most affected by the presence of thiostrepton.
Their ability to translate was inhibited at a 1 μM concentration of thiostrepton and nearly
abolished at 2 μM. The concentration at which the ribosomes retained 50% of their
initial ability was estimated to be 1.5 μM. The mutant strains were less susceptible to the
antibiotic; however, at higher concentrations, thiostrepton did affect their translation.
The N-terminal minus, NVD002, ribosomes retained 50% of their initial translation
ability at 3.5 μM of thiostrepton. The L11 minus, NVD005, ribosomes were the most
resistant to the effects of higher thiostrepton concentrations. They retained 50% of their
initial translation ability at 4.5 μM. However, even the mutant ribosomes lost nearly all of their function at 10 μM thiostrepton.

Stoichiometric concentrations (approximately 1 μM) of thiostrepton and ribosomes affected the wild type, NVD001, ribosomes the most, revealing the importance of the L11 protein for tight binding of the antibiotic. Past studies have shown that thiostrepton binds ribosomes extremely tightly with a $K_d$ of $2.4 \times 10^{-7}$ M (Thompson and Cundliffe 1991). This evidence helps explain the concentration and extent at which thiostrepton inhibits translation of wild type ribosomes.

As mentioned above, thiostrepton was dissolved in DMSO, which has a stimulatory effect on translation. It is hypothesized that DMSO favors factor interactions with the ribosome and makes it more susceptible to conformational changes occurring during translation, possibly enabling the ribosome to translate better. Regardless of the mechanism, the effects of DMSO were visible in the studies presented, especially in the translation by the wild type and the NVD005 ribosomes. Upon examination of the data presented in Figure 2.3, small concentrations of thiostrepton appear to stimulate translation with these two strains. However, it is likely that the stimulation seen is due to the DMSO present in the translation reaction. Control experiments were done that showed similar stimulation with the addition of small amounts of DMSO present in the translation mixture (data not shown). The most valuable information that can be obtained from these data was the concentration at which the ribosomes lost a large portion of their translation ability. These data are reproducible and consistent with previous research in the field.
Spectinomycin

The second antibiotic used to functionally characterize the L11 mutant ribosomes was spectinomycin. As mentioned above, spectinomycin is also a translocation inhibitor; however, it binds in the 30S subunit. Mutations causing resistance arise in protein S5, which is located on the small subunit, not the large subunit where the GTPase center and the L11 protein are located. Spectinomycin does not bind the ribosome with the same affinity as thiostrepton. Its extent of inhibition was only about 50 to 60% compared to translation without the antibiotic. Spectinomycin was dissolved in water. The inhibition pattern for all three strains of ribosomes was very similar. These results show that the
function of spectinomycin, which binds in the 30S subunit, is not affected by the presence or absence of the L11 protein.

Figure 2.4: In vitro Translation with Spectinomycin. Translation with NVD001 ribosomes is shown in blue, NVD002 ribosomes in magenta, and NVD005 ribosomes are yellow. Ribosomes are inhibited similarly. 022 mRNA was used. Error bars represent the standard deviation.

Streptomycin

The last antibiotic used for functional characterization was streptomycin.

Streptomycin causes translational misreading and is not thought to be a specific translocation inhibitor. Mutations in protein S12, and in the 16S rRNA have been shown to lead to resistance (Springer, Kidan et al. 2001). These regions are not associated with the L11 protein; however, streptomycin has been shown to stimulate GTPase activity (Novogrodsky 1971). This indirect relation to the L11 protein warranted the following experiments to better characterize the series of mutants.
Similar to spectinomycin, streptomycin, at low concentrations, inhibited ribosome function to about 50%. In fact, the L11 mutant ribosomes, NVD002 and NVD005, reached a 50% inhibition at .5 μM and that level of inhibition remained constant throughout the titration of increasing streptomycin concentration. The wild type ribosomes responded differently. They too reached approximately a 50% inhibition at 0.5 μM; however, increasing the streptomycin concentration over this level improved \textit{in vitro} translation. At 100 μM streptomycin, only a 15% inhibition was observed. The data are shown in Figure 2.5.

\begin{figure}[H]
\centering
\includegraphics[width=\textwidth]{figure2.5.png}
\caption{\textit{In vitro} Translation with Streptomycin. NVD001 ribosomes are shown in blue, NVD002 in magenta, and NVD005 in yellow. NVD002 and NVD005 ribosomes were affected similarly, but NVD001 ribosomes were affected differently by higher concentrations of streptomycin. 022 mRNA was used. Error bars represent the standard deviation.}
\end{figure}
Discussion

Mutations in the L11 protein dramatically affect ribosome function. This is evident in the growth rates of the different stains of *Escherichia coli* used for these studies. It is also evident in the *in vitro* translation data presented. Because the L11 protein is part of the GTPase center and it has been implicated as part of an antibiotic binding region, characterization of L11 mutants in the presence of these antibiotics warranted detailed consideration. *In vitro* translation offered a measure of their function in the presence of three antibiotics.

Thiostrepton

Footprinting of thiostrepton has mapped its binding region to the GTPase center. Furthermore, mutant bacteria that have acquired resistance to thiostrepton have transversion mutations at A1067 and A1095. Some resistant strains lack the N-terminus of L11 or the entire protein. The results presented here concur with previous structural work. The wild type strain is most sensitive to the antibiotic, followed by NVD002, the N-terminal minus strain, followed by NVD005, the L11 minus strain.

Thiostrepton binds ribosomes very tightly, with a $K_d$ of $2.4 \times 10^{-7}$ M (Thompson and Cundliffe 1991). The effects of tight binding were apparent during translation. At stoichiometric amounts of thiostrepton, wild type ribosome translation was severely inhibited. This was not as evident in the two mutant strains because they had an inherent level of thiostrepton resistance.

Previous studies of thiostrepton inhibition have suggested that the N-terminal domain is very flexible and involved in a conformational change during factor
interactions. In contrast, the C-terminal domain is relatively tightly bound to the rRNA in the same region. Thiostrepton was hypothesized to lock the N-terminus and not allow the conformational change to occur, inhibiting the function of translation factors associated with the L11 protein (Wimberly, Cell, 1999). If the N-terminus of L11 has been deleted, like the NVD002 strain, or if the entire L11 protein is missing, like the NVD005 strain, the thiostrepton binding region is disturbed. However, thiostrepton likely still has access to the rRNA binding region, so that it can still inhibit ribosome function at higher concentrations.

The two mutant strains, NVD002 and NVD005, were affected similarly by thiostrepton, but the NVD002 ribosomes were more sensitive to the antibiotic. Because these ribosomes still have the C-terminal domain, it is likely that the structure of rRNA is more similar to the wild type. Although the entire protein is not present, the C-terminal domain may at least partially stabilize the region. This is especially true, because of the nature of the C-terminus interaction with the rRNA. Its tight binding, mentioned above, should preserve the structure and nature of the thiostrepton binding region much better than L11 minus ribosomes could.

Spectinomycin

Previous studies of spectinomycin show its binding site to be in the 30S subunit of the ribosome. Resistance to spectinomycin is related to mutation in protein S5 and nucleotides in the 1060 and 1192 regions in helix 34. It was chosen because it is known to be a translocation inhibitor, like thiostrepton, but it has a different binding site. The effects of spectinomycin were very similar in the three different strains of bacteria. It
does not bind the ribosomes with the affinity that thiostrepton does, and the inhibition of translation was found to be about 50%.

Streptomycin

The results of streptomycin inhibition provide interesting insight into the necessity of the L11 protein. The observation of a partial rescue of translation in wild type ribosomes contrasted the consistent inhibition of both N-terminal minus and L11 minus ribosomes. It has been known for many years that streptomycin leads to error-prone ribosomes. Early data suggested that the proof-reading step was affected (Ruusala and Kurland 1984), and additional studies suggested that the initial selection of tRNAs and the proof-reading steps are affected by streptomycin binding (Bilgin and Ehrenberg 1994; Karimi and Ehrenberg 1994; Karimi and Ehrenberg 1996). Crystal structures of the 30S subunit with streptomycin show that it is tightly bound to the phosphate backbone of 16S rRNA; it contacts K45 from protein S12 (Carter, Clemons et al. 2000). These models suggest that streptomycin may also contact nucleotides U14, C526, G527, A914, C1490, and G1491. Streptomycin resistance arises with mutations in the S12 protein and in 16S rRNA.

Although streptomycin contacts appear to occur in these mentioned regions, previous work has shown that streptomycin stimulates GTPase activity (Novogrodsky 1971). This information implies that it has either direct or indirect effects on the GTPase center, and possibly the L11 protein. Another study examined the binding site of streptomycin through photoaffinity labeling experiments (Abad and Amils 1994). Their data imply that streptomycin is in close contact with L11 and L23, and other small
subunit proteins. Additional data on the synergistic nature of GTPase activity with EF-Tu and EF-G suggest a stimulatory effect of streptomycin and an inhibitory effect of thiostrepton and other antibiotics (Mesters, Potapov et al. 1994).

The data presented in this paper suggest that streptomycin exhibited partial stimulatory effects, but only in the wild type ribosomes. The increased protein synthesis may be due to several different mechanisms. It is understood that streptomycin binding induces the ribosomes to be more error prone. The presence of errors in the *in vitro* translation experiments presented would not be detectable. As long as the errors specified tRNAs that were available in the translation mixture, an error-rich peptide synthesized at a faster rate would appear in *in vitro* translation assays to represent an increased level of translation.

This explanation does not describe why the ribosomes would translate faster, or why the mutant ribosomes did not present the partial rescue mechanism visible in wild type ribosomes. Increased GTPase activity could explain the apparent discrepancy. If streptomycin does stimulate GTPase activity, as it were in previously described studies, the ribosomes could synthesize a greater amount of peptide. Furthermore, it appears that this stimulation is dependent on the L11 protein, and not only the C-terminus of the L11 protein. It follows that the N-terminus is either directly involved in the stimulatory effect, or necessary to influence/stabilize another region of the ribosome to be amenable to the streptomycin effect. This interaction may involve a series of dynamic changes if streptomycin only makes contact with the 30S subunit. The effect would have to cross the subunit interface to affect the GTPase center and L11 protein.
Further examination of the \textit{in vitro} translations comparing all combinations of ribosomes and s100s (see figure 2.2) highlights several curious results. The ability to translate would be expected to correlate with the growth rate. However, the NVD005 strains appeared to translate better than expected. If the wild type translation is compared with the NVD005 translation, each strain's ribosomes with their own cell lysate, or s100 fraction, the wild type strain translates approximately 4 times as well. This is different from the apparent growth rates, where the wild type strain grew approximately 6 times faster than the L11 minus strain. Comparison with the NVD005 s100 with both ribosomes yields approximately a 6-fold difference, which coincides with growth rates. This evidence suggests the stimulation to be a direct result of the NVD005 s100.

Perhaps the most striking feature of the graph is this difference in translation comparing the various s100 fractions. The NVD005 s100 appeared to allow a much greater extent of translation than the other s100s, especially when in combination with wild type, NVD001, ribosomes. These data may help to explain the discrepancy between growth rate and translation, along with more knowledge about the stimulation of translation. To further examine the effect of the s100 fractions, additional experiments were conducted that tested more specific details of translation.

Many of these experiments were done using only wild type ribosomes, and comparing the effects of wild type and NVD005 s100s. The options for the composition
of the mystery factor(s) in the NVD005 s100 are numerous. It may be made up of protein, RNA, DNA, or some combination of these possibilities.

*In vitro* translation of wild type ribosomes was conducted with wild type and NVD005 s100s after they were treated with proteinase K, to eliminate proteins in the lysate. If a protein were the direct cause of stimulation in the NVD005 s100, a decrease in stimulation would be expected. 50 µL of s100 were treated with 1.25 µL proteinase K (diluted 10 times with NB) and incubated at 37°C for 90 minutes. The s100s were then phenol:chloroform extracted to remove the remaining proteinase K. In the translation experiments, 2 µL of treated s100 (both wild type and NVD005) were combined with 2 µL of untreated, wild type s100 so that necessary proteins were available. In the controls, 4 µL of untreated s100 (wild type and NVD005) were used for comparison. The results are shown in Figure 3.1.

![Translation with Proteinase K treated s100](image)

Figure 3.1: Translation with Proteinase K treated s100. Proteinase K treatments of NVD001 and NVD005 s100 were compared using wild type ribosomes. Untreated s100 are shown in blue, and treated s100s are shown in red. Experiment done in duplicate, averages are shown.
Clearly, the proteinase K treatment had severe effects on both wild type and NVD005 s100s. It was clear that the proteinase K digestion of the s100s, both wild type and NVD005, caused both to lose a lot of their ability to translate when used in the *in vitro* translation system. However, the proteinase K and phenol:chloroform extraction treatment is very harsh on these labile s100 fractions. It is so harsh that the ribosomes were not able to translate as well as they would in only 2 μL of either s100. Therefore, making strong conclusions from this evidence is difficult. The protease treatment does appear to remove the stimulatory factor in the NVD005 s100; however, it also decreases the general translation ability. Therefore this experiment suggests that the mystery factor does have a protein component, but it is not conclusive.

To better characterize the stimulation of the NVD005 s100, kinetics data were obtained. These experiments were designed to offer insight into the stage of translation that is affected. For instance, we can learn whether the stimulation occurs at the beginning of translation, or if there is a lag time before the stimulation occurs. To obtain kinetics information, translations were conducted similar to that described in the Materials and Methods of Chapter 2, but each translation was stopped at specific time points. The amount of peptide synthesized was quantified as described previously. All kinetics experiments used separate reactions for each time point. They were not drawn from one reaction. This protocol may have led to more variability, but it was less likely to offer an entire artifactual test. The following experiments used only wild type ribosomes, but both wild type and L11 minus, NVD005, s100s. The *in vitro* translation data are shown in Figure 3.2.
Figure 3.2: Kinetics Translations with 022 mRNA.
These data show wild type (NVD001) ribosome translating with NVD001 s100 (blue) and NVD005 (magenta). The top graph spans 60 minutes, whereas the bottom graph only spans 300 seconds. Each data set is from one experiment.
The kinetics data help to understand when the stimulation of translation occurs. The data shown with the mRNA suggests that the stimulation occurred nearly immediately after translation begun. Ideally, we would like to obtain initial rates of translation, to more precisely monitor the effects of the different s100s. However, data suggest that in vivo, ribosomes can translate an mRNA at approximately 10 amino acids per seconds. In vitro rates may be slightly slower, depending on conditions, but because the mRNA used is only 19 amino acids long, the ribosomes may have translated several mRNAs before the first time point occurred. Also, the mRNA in vitro translation system is sensitive to all stages of translation, so it is hard to discern whether any stages are being affected more than others by the NVD005 s100.

To gain a better understanding as to which step(s) of translation are affected most by the s100 component, we used another variation of in vitro translation. This translation utilized a poly-uridine (poly-U) mRNA instead of the synthetic mRNA. This RNA is made up entirely of uridine nucleotides. The UUU codon encodes phenylalanine, and therefore the peptide synthesized will be poly-phenylalanine. In this assay, it is not necessary to add initiation factors to the mixture; all of the essential factors come from the s100 fraction. Because no AUG start codon or any stop codon exists, the ribosomes do not initiate and terminate translation as they do on a normal mRNA. They simply translate until they fall off of the poly-U RNA. In essence, the system provides insight into the elongation ability of the bacteria, as they are only elongating the peptide. This system is very simplified and is even further removed from in vivo conditions as compared to the synthetic mRNA system described. However, when this approach is used understanding its narrow parameters, it can provide very useful information.
In vitro Poly-U translation requires many of the same components as the previous in vitro translation discussed except for the exogenous initiation factors. In this system, it is still necessary to have ATP and GTP to assist the elongation factors. MTHF is not necessary, because there are no methionines that need to be formylated to initiate translation.

Figure 3.3: Poly-U Translation wild ribosomes and two s100. NVD001 s100 is shown in blue, and NVD005 s100 is shown in red. Experiment done in duplicate, averages are shown.
Figure 3.4: Poly-U *In vitro* Translation Kinetics. NVD001 s100 is shown in blue, and NVD005 s100 is shown in red. Both s100 were in combination with wild type ribosomes. The data set represents one experiment.

A kinetics experiment examining in vitro translation was conducted with poly-U mRNA, as shown in Figure 3.4. This experiment utilized only wild type ribosomes, and again compared the s100 fractions from wild type and NVD005 ribosomes. A curve was constructed from the various time points for each s100. The latter time points appear to show similar data to that from the 022 synthetic mRNA. That is, the wild type ribosomes were able to translate more with the NVD005 s100 than with the wild type s100.

In these kinetics experiments, translation was stopped at 30 seconds, 60 seconds, 90 seconds, 300 seconds, 600 seconds, and 1200 seconds. As with the mRNA, these experiments show that the stimulation of translation occurred very early, if not immediately, and lasted throughout the duration of translation.
Figure 3.5 shows a close up of two independent experiments, looking at only the first 90 seconds and 120 seconds of translation, respectively. We crudely compared initial rates of elongation with this data, examining the slope of each curve. It is necessary to look at the early time points, because soon after translation begins, the ribosomes start multiple rounds of translation, and the elongation rate that could be determined from poly-U translation becomes mixed up with other occurrences, including the recycling of the ribosome, location and binding of the poly-U, and many other events associated with translation. Both graphs are shown to gain a better understanding of the situation. The first graph shows a small, slightly upward sloping difference in initial rates, suggesting the NVD005 s100 may have a slightly higher elongation rate. In the second graph, the initial rates appear to be nearly identical.

If we do assume that initial rates of these kinetics graphs, elongation rates, are equal, we can rule out the elongation step as being stimulated by NVD005 s100. Therefore, we would have to examine the sensitivity of initiation, termination and recycling of the ribosome.

As mentioned above, in vivo rates imply that it would be nearly impossible to obtain initial rates of translation via human pipetting, even if poly-U RNAs average 300 nucleotides in length. If the ribosomes translate half of the poly-U RNA (there is no start codon, so initiation of the RNA occurs randomly) and translate to the end, recycling of the ribosome may occur on average after only 15 seconds, assuming ribosomes are translating as fast as in vivo condition. Furthermore, the results are not vivid, and could be interpreted either way. On the other hand, these early time points are most heavily influenced by the elongation step of translation, rather than the efficiency of recycling,
initiation of the poly-U, etc. Therefore, estimations can be made, but with careful consideration and understanding of the system.

![Poly-U Kinetics 11/12/02](image)

**Figure 3.5: Poly-U Translation Kinetics.**
Wild type ribosomes translated with NVD001 s100 (blue) and NVD005 s100 (magenta) in two independent experiments. These graphs help examine initial rates of elongation. Each data set represents one experiment.
To examine the initiation step of translation, we used the in vitro translation with the synthetic mRNA, and varied the amount of exogenous initiation factors added to the mixture. This translation was carried out with wild type ribosomes and wild type and NVD005 s100s. This system would provide insight if one s100 enabled the ribosomes to better cope with a lesser amount of exogenous Initiation Factors. The results from several independent experiments are shown in Figure 3.6. These graphs are shown separately, rather than compiled into one, to provide a more comprehensive understanding of the results.

Each graph shows translation with time points, 90 seconds, 5 minutes, 10 minutes and 20 minutes, to help monitor the effect. Translation was done with either no exogenous initiation factors or 1.5 μM initiation factors added to the ribosomes mixture. Ribosomes were present at 3 μM in the ribosomes mixture, so these levels are substoichiometric. This is significant, because at substoichiometric levels, the ribosomes will be limited in their ability to initiate translation. If no exogenous initiation factors are added, the ribosomes would be completely dependent on the s100 that is added to the translation system. At 1.5μM IFs, the ribosomes will still be deficient in the factors needed to initiate translation, but not as limited as without any IFs. The following experiments offer insight into whether the s100 can enable the ribosomes to cope better in deficient conditions.
Figure 3.6: *In vitro* Translation with Initiation Factor Titration.
NVD005 s100 with 1.5μM IFs (light blue), NVD005 s100 with no IFs (yellow), NVD001 s100 with 1.5μM IFs (magenta), and NVD001 with no IFs (dark blue) translated with wild type ribosomes. NVD001 s100 with 1.5μM IFs appeared to allow less translation than NVD005 s100 at 1.5μM IFs. Without IFs, both s100s responded similarly. Each data set represents one experiment.
NVD005 s100 enabled the ribosomes to better cope with substoichiometric levels of Initiation Factors in all three experiments. From the latter two graphs, it appears that the NVD001 s100 with 1.5 μM IFs allowed the ribosomes to translate better than either s100 without any exogenous IFs. In our ribosome purification protocol, the ribosomes are salt washed so that the IFs can be added back with known amounts. If the only source of IFs were the s100s, their ability to enable wild type ribosomes to translate should be limited, as depicted in figure 3.6.

The results suggesting that NVD005 s100 allows the ribosomes to cope with some IFs (1.5 μM), although substoichiometric, better than the wild type s100 offers insight into the mechanism of function. This adds further credence to the previous poly-U translation data that suggest that it is not the elongation step that is being affected. It suggests that either the initiation step is either directly affected by the stimulatory effect of NVD005 s100 or the termination/recycling step is affected and more efficiently prepares the ribosomes to initiate more translation.

Another experiment examining the stimulatory effect was conducted with varying amounts of mRNA. Again we can examine whether the different s100s allow ribosomes to translate in varied conditions. In the case of substoichiometric mRNA concentrations, results offer insight into the ability to recycle mRNA for translation. Superstoichiometric levels of mRNA may help compensate for a possible hindered ability to recycle mRNA. Wild type ribosomes were at the standard in vitro translation concentration of 1 μM. The substoichiometric concentration of mRNA was 0.35 μM, and the superstoichiometric concentration was 1.5 μM. This experiment aimed to investigate the initial stages of translation and how the ribosomes processed varying amounts of mRNA in the presence
of wild type and NVD005 s100. Kinetics time points were taken at 10 seconds, 30 seconds, 60 seconds and 90 seconds.

![Sub/Super Stoichiometric mRNA]

Figure 3.7: *In vitro* Translation with sub/superstoichiometric concentrations of mRNA. Both NVD001 s100 and NVD005 appeared to respond similarly at a substoichiometric concentration of mRNA (blue and magenta, respectively). At superstoichiometric levels of mRNA, NVD005 (light blue) enabled more translation than NVD001 (yellow). The data set represents one experiment.

The ribosomes appear to translate equally well with the two different s100s at substoichiometric mRNA concentrations. However, at superstoichiometric mRNA concentrations, the NVD005 s100 allows the ribosomes to translate to a greater extent. These data suggest that stimulatory effect of the NVD005 s100 may have to do with the efficiency of recycling mRNA. The ribosome has to bind the mRNA during the initiation step of translation. It also has to dissociate from the mRNA after it reaches the stop codon, and the protein is complete. This evidence again suggests that either the initiation step or the termination/recycling step of translation is affected by the stimulatory factor(s) in NVD005 s100.
One last experiment was conducted with *in vitro* translation to further examine the initiation step of translation. Exogenous 30S ribosomal subunits (mre600 strain) were added to mRNA *in vitro* translation and the concentrations of Initiation Factors doubled (6μM in ribosomes mixture). This addition would enable the formation of additional initiation complexes. Doubling the IFs would enable the ribosomes to take advantage of the exogenous 30S subunits. This experiment was constructed to try and examine whether the initiation step was the determining step for the stimulation from NVD005 s100. If the wild type s100 could not enable initiation as well as NVD005 s100, it may be compensated for in these experiments. Kinetics time points were taken at 10 seconds, 30 seconds, 60 seconds, 90 seconds, 300 seconds, 600 seconds, and 1200 seconds. The results are shown in Figure 3.8.
Examination of the kinetics over 20 minutes yields varying results. In the case of 3 μM IFs, the NVD005 s100 appeared to enable more translation with and without exogenous 30S subunits. In the presence of 6 μM IFs and exogenous 30S subunits, both s100s performed equally. This may suggest that NVD005 s100 enabled ribosomes to initiate more efficiently because the added initiation complexes helped the wild type s100 enable more translation. However, when comparing this evidence with the control graph (no exogenous 30S subunits), the translation with NVD005 s100 appeared to be decreased, rather than wild type s100 translation increased.

Initial rate comparisons suggest that ribosomes coupled with exogenous 30S subunits are very similar in the presence of both wild type and NVD005 s100s. Yet in the control graph without exogenous 30S subunits, the NVD005 s100 appears to have a faster initial rate of translation which is in agreement with previous assessments of initial
rates. Similar to the long term kinetics described above, it appears as if the NVD005 s100 translation was decreased, rather than wild type s100 translation increased. These results suggest once again that the initiation step of translation is directly or indirectly affected by the stimulatory effect of NVD005 s100; however, they are inconclusive as to the precise mechanism of action or the precise step of involvement.

These variations of in vitro translation help to better characterize the interesting stimulatory nature of the NVD005 s100. They allow us to crudely tease apart different steps of translation; however, they do not specifically suggest a mechanism or offer an explanation. In combination with the proteinase K experiment, we are led to believe that it may be a protein that is causing these effects. Because many of the translation factors are GTPases, and interact with the L11 protein, we have a list of likely candidates, including IF-2, EF-Tu, EF-G, RF-3, and RRF. Other release factors also interact in this region as mentioned in the introduction. Poly-U data and initiation sensitive experiments suggest that the elongation step is not being affected, so we may be able to narrow our list to IF-2, RRF and the release factors, or any combination. In fact, we cannot rule out the elongation factors, because they may also act in combination with other factors to stimulate translation. Overall, we have a better understanding of the stimulation, yet further experiments are necessary to better characterize the mystery factor(s).

RNA Possibility

It is also possible that a protein is not the only cause of the stimulation, or perhaps it is another component entirely. The wild type and NVD005 s100s were tested early on to see whether one had more RNase activity than the other. This would be a simple
explanation to the stimulation seen with the NVD005 s100. The experiment was conducted by incubating the different s100 fractions with mRNA. Then the samples were run on a 8% polyacrylimide gel and stained with ethidium bromide. Neither mRNA appeared to be degraded; however, the NVD005 s100 did reveal two novel bands on the gel. See Figure 3.9. No conclusive data have been obtained that would relate either of these bands to the stimulatory effect of the NVD005 s100; however, it does show one more difference between the two s100s and offer another crude but possible explanation.

![Figure 3.9: 8% Polyacrylamide stained with Ethidium Bromide.](image)

This gel qualitatively examined the RNase activity of NVD001 s100 and NVD005 s100. Two additional bands are visible in NVD005 s100 and may be a component of the stimulatory factor in NVD005 s100.

More recent studies have shown the involvement of the L11 protein in the stringent response. The stringent response was discovered in the late 1960’s with the discovery of (p)ppGpp, magic spot, which is a modified guanine nucleotide (Cashel 1974; Cashel 1975; O'Farrell 1978). As described in the introduction, the L11 protein is intimately related to the production of (p)ppGpp.
Recent results from collaboration with our lab have shown ppGpp to stimulate protein synthesis in certain conditions (Scott P. Hennelly, unpublished). This response suggested the hypothesis that an increased ability to produce (p)ppGpp may be the cause of the stimulation from the NVD005 s100.

To assay for the amount of ppGpp produced, a mixture was made with 2 μM ribosomes, 4 μL s100, 2 mM GTP and 4 mM ATP and radiolabelled γ-ATP. This reaction was incubated at 37°C for the specified amount of time. Experiments were also prepared with ribosomes in the identical situation as poly-U translations. Some of these reactions were prepared without amino acids to try and encourage the stringent response to be activated. After the incubation, 2.5 μL of each reaction were spotted onto PEI-cellulose Thin-Layer Chromatography (TLC) Paper. Radiolabeled γ-ATP and cold ppGpp were run as standards. Experiments were also run with 32P-labeled α-GTP and cold ATP. The TLC paper was placed in 3.5 M Potassium Phosphate (pH=3.5) and allowed to run in a sealed chromatography tank. The paper was dried, developed for 4 hours or 12 hours and then analyzed with the Fuji Phosphoimager. ppGpp was visible under a short wave UV lamp. Theoretically, all species of nucleotides should be visible under the UV lamp, but due to the small concentrations of each, only the standards were visible. The standards were compared to the autoradiographs, and analyzed. The results are shown in Figure 3.10.
Figure 3.10: Autoradiograph of TLC.
The production of ppGpp using NVD001 s100 and NVD005 s100 is shown at different time points. The control lane of ppGpp, marked with an oval, was visualized using a short wave UV lamp and transposed onto the autoradiograph. The TLC was trimmed just below the band of radiolabeled free phosphorous.

There appears to be a spot on the TLC that measures up with both a spot in the control γ-ATP lane and with the pure ppGpp. Experiments with radiolabeled α-GTP revealed similar results. There is a distinct variation in the amount of this spot comparing the production with wild type s100 versus NVD005 s100. If this spot corresponds to ppGpp, and considering the results that ppGpp stimulates translation in certain circumstances, these data may provide evidence that ppGpp is the mystery factor causing stimulation in the NVD005 s100. There are many precautions that must be taken with this hypothesis. There is no definitive evidence that this spot corresponds to ppGpp. Mass spectrometry data was obtained on these spots, and there was too little of the purified compound to be distinguished from background readings. Furthermore, we need
to obtain more data about how ppGpp stimulates translation for this to be a feasible explanation.

The results presented in this chapter offer more insight into the mystery factor(s) in NVD005 s100 that caused a stimulation of translation. More data need to be acquired so that the cause of stimulation can be elucidated. The evidence provided together with further study can offer a better understanding of the L11 protein and its function in translation, as well as response mechanisms related to translation and ribosome function.
Chapter 4
Conclusions and Future Prospects

The studies described in this thesis provide a functional characterization of ribosomal protein L11 mutants. Growth characteristics were compiled based on the doubling times for the wild type, N-terminal minus (NVD002), and L11 minus (NVD005) strains. The NVD002 strain grew approximately twice as slow and the NVD005 strain grew approximately six times as slow as the wild type strain of bacteria.

In vitro translation was also used to characterize the ribosomes and the cell lysates, s100 fractions, from each strain. The translation data did not correlate well with the growth rate data; the NVD005 ribosomes were able to translate better than expected. Furthermore, the NVD005 s100 fraction had a stimulatory effect on translation, especially in combination with wild type ribosomes.

The ribosomes were characterized in the presence of three different antibiotics: thiostrepton, spectinomycin, and streptomycin. Thiostrepton binds in the GTPase center of the ribosome with high affinity and inhibits translocation. Its binding likely is stabilized by the N-terminal domain of L11, and the 1067 and 1095 step loops of 23S rRNA. Thiostrepton affected wild type ribosomes the most, followed by NVD002 ribosomes, and least affected were the NVD005 ribosomes. These data correspond with conditions in which mutations in the L11 protein have led to thiostrepton resistance. Spectinomycin was used because it is also a translocation inhibitor, but it binds in the 30S subunit. As hypothesized, it affected all three strains of ribosomes similarly. Streptomycin affects the fidelity of translation and also binds in the 30S subunit. It has
been shown that streptomycin and spectinomycin often have opposite effects between sensitivity and resistance. Streptomycin also has been shown to have stimulatory effects on the GTPase activity. This antibiotic inhibited ribosome translation similarly in all three mutants at low concentrations. However, at higher concentrations, and only in wild type ribosomes, there appeared to be a rescue of translation, restoring much of the translation ability. It follows that the rescue mechanism necessarily requires function of the L11 protein, and possibly stimulates GTPase activity to help translation.

Variations of in vitro translation were used to better characterize the stimulatory effect of NVD005 s100. Poly-U translations, Initiation Factor Titrations, Sub/Superstoichiometric mRNA, and Kinetics point to either the initiation step or the termination/recycling step of translation as a source of stimulation as opposed to the elongation step. Experiments with proteinase K imply that there may be a protein component to the mystery factor in NVD005 s100, although the data are not definitive. Additional experiments imply that maybe there is an RNA component to the mystery factor.

The close relation of the L11 protein to the stringent response suggests another possibility in explanation the stimulatory nature of NVD005 s100. Unpublished data suggests that in certain circumstances, ppGpp provides a stimulation to in vitro translation. Thin-Layer Chromatography experiments suggest that there is an increased ability to produce ppGpp with the NVD005 s100 compared to wild type s100. These results are also not definitive but imply a difference in RelA or SpoT concentrations in the two s100 fractions.
Future experiments may help to elucidate the mystery factor in NVD005 s100. Present work with ppGpp and stringent response is promising and should continue. Western blotting may be useful to determine the amount of RelA, SpoT and possibly other proteins in the different s100s. Although experiments described in Chapter 3 have led us to believe it is not the elongation step of translation that is being affected, it would be useful to examine all of the translation factors for signs of up or down regulation. RNase protection assays may be useful for these experiments. Also a mini gene chip experiment looking for hybridization of all of the factors related to translation may provide results suggesting differences in the various s100s. Logic would suggest that the mystery factor would be closely linked to the L11 protein. It may be one or more of the GTPase factors in translation. This knowledge combined with the evidence presented in Chapter 3 suggests a GTPase protein involved with either initiation or termination/recycling or with the stringent response. The stimulation may be due to any combination of these or any other of the components present in the s100.

These experiments may provide even more insight into the importance of the L11 protein on the 50S subunit of the ribosome. Ribosomes can function without the protein, and in the presence of certain antibiotics, they even appear to have an advantage. The L11 protein certainly has an integral role in ribosome function and specifically with the GTPase center. Cells growing without the L11 protein are stressed enough to induce a response mechanism that allows increased translation. Although the precise elucidation of the stimulatory mechanism remains a mystery, the characterization of L11 mutants offers more insight in the function of the ribosome.
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