Elucidating ribosomes-genetic studies of the ATPase Uup and the ribosomal protein L1

by

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A dissertation submitted in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy
(Molecular, Cellular, and Developmental Biology)
in the University of Michigan
2015

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Dedicated to:
My parents, without whom I would never have embarked on this journey, and without whose support I would never have finished;
My daughter, who has had to do without me more than she would like, and who has motivated me to press on;
My family, whose love and support I treasure;
And those dear friends whose prayers and encouragement carried me on this journey.

Acknowledgments

I would like to begin by thanking my PhD advisor, Dr. Janine Maddock, for all her support, both personally and professionally. Janine has not only mentored me as a scientist, but has also been a powerful encourager and advocate for me as I ventured to make personal choices and navigated the consequences of those choices. Without her care and flexibility, I would not have continued in my pursuit of this PhD. She has provided an example for me in the way she vehemently cares for individuals, to a degree that far exceeds the requirements of her job. Janine's support has changed my life for the better, and I am extremely grateful.

Likewise, I am indebted to Dr. Carlos Gonzalez, whose kind teaching and belief in me motivated me to dare to aspire to this path, and who first directed me to Michigan. It was in his lab I developed an interest in pursuing research science. I would not have attempted this without his confidence in me.

I am also thankful to the excellent post-doctoral researchers who have taught and encouraged me while in the Maddock lab. I thank Dr. Sonia Bardy for "showing me

the ropes" and providing a solid base in many of the techniques and lab skills I used in the course of my research. I thank Dr. Richard Zielke for his sense of humor and the sense of belonging he shared with me, and for his foundational work on the topics discussed in this thesis. I thank Dr. Raji Janakiraman for the commiseration we shared, and for keeping me company during the late nights we spent in lab together working on our lab reports.

I especially thank Dr. Lolita Piersimoni for her spunk, ready smile, and humor that helped make the darkest days brighter, and for her significant contributions to my professional development. She has generously shared her time in many discussions to help me develop and test my ideas as well as in lending her much more able hands in helping me to obtain many "just one more" pieces of data. Our many discussions have been invaluable in my journey to independent thinking worthy of a PhD. In many ways, she has been an older sister to me in this journey.

I would also like to thank all the other Maddock lab members I have known while here, especially Lance Shultz and Dr. Billy Clifford-Nunn. Your friendship has meant a great deal to me.

The members of my committee, Dr. Matt Chapman, Dr. Lyle Simmons, and Dr. Nils Walter have all been extremely helpful in my journey. Possibly consciously, probably unconsciously they have all in turn been the one smiling face to give me hope and the grim disciplinarian to require me to keep aspiring higher. I have found

courage in hope, and am glad of what their sternness helped goad into development. I appreciate their faith in me, and their genuine good-will.

I would also like to thank the members of the wider MCDB community who have touched me during my time here. In particular, I thank Dr. Mohammed Akkabounne for taking me in and teaching me neurobiology, Mary Carr for being a knowledgeable and dependable resource, my classmates, particularly those with whom I have kept in contact, for being a kind and caring support group as we all adjusted to this new venture, and members of the micro-bio "Super Group" for their many intellectual contributions to my work.

A special mention goes to Dr. Marcus Ammerlaan whose passion for teaching and keen sense of humor have been refuges for me when research felt overwhelming.

Finally, I would like to thank my family, particularly my parents, grandparents and siblings, for closing ranks around me during this time. Your support has been another indispensible help in completing this degree. You have all done much more than I could ask to help me succeed, and I cannot thank you enough. I also thank my daughter for gladly becoming part of Mommy's science life, and generously sharing me with this PhD. Your smile and the knowledge that the outcome of this degree program is vitally important in your life as well as in mine have motivated me when nothing else would have mattered.

Thank you to all who have walked this road with me, to all who have lit the way, to all who have cheered me on. Thank you to all who have helped me walk another little while, and to all who have carried me when I was too weak to keep going on my own. This is your victory as well as mine.

Table of Contents

Dedication ii

Acknowledgements iii

List of Tables ix

List of Figures x

Abstract xii

Chapter

1. **Introduction**
    - **The bacterial ribosome** 1
    - **Ribosomal protein L1** 3
    - **Ribosomal Protein L11** 7
    - **Translation** 8
        - **Initiation** 8
        - **Elongation** 11
        - **Termination/Recycling** 12
    - **Elongation Factors** 14
        - **GTPase elongation protein family** 14
            - **EF-Tu** 15
            - **EF-G** 18
            - **LepA** 20
            - **BipA** 22
        - **Non-GTPase bacterial elongation factors** 24

EF-P 24
EttA 26
References 27

2. Uup is an ATPase involved in translation 49
Introduction 49
Methods 54
Results 59
Discussion 70
References 76

3. Characterization of a Δ*rplA* mutant of *E. coli* 97
Introduction 98
Methods 100
Results 102
RpoS regulation/regulon 105
Ribosome/translation connected suppressors 110
DNA replication-involved suppressors 115
Proteins of unknown relevance 116
Discussion 118
References 121

4. Future Directions 141

List of Tables

Table 2.1 Strains and plasmids (used in Chapter 2) 80

Table 3.1 Strains and plasmids (used in Chapter 3) 130

Table 3.2 Functional classes of ΔL1 slow growth in the cold high copy suppressing proteins 136

List of Figures

Figure 1.1 Features of the large ribosomal subunit 43

Figure 1.2 Initiation of translation involves formation of a 30S pre-initiation complex and subsequent joining of the 50S subunit 44

Figure 1.3 EF-G catalyzed translocation 45

Figure 1.4 Release Factors 1 and 2 interact with the stop codon in the A-site of the ribosome 46

Figure 1.5 Domain structure of the elongation GTPase family 47

Figure 1.6 E-site translation factors 48

Figure 2.1 Uup is a Δ*bipA* suppressor 82

Figure 2.2 Growth consequences of plasmid-borne mutant Uup in presence and absence of endogenous Uup 83

Figure 2.3 Growth consequences of plasmid-borne mutant Uup on strains lacking BipA 84

Figure 2.4 Dominant negative Uup variants associate with ribosomes more strongly than other variants 85

Figure 2.5 Uup is found on ribosomes in vitro and in vivo 86

Figure 2.6 Overexpression of Uup results in earlier correction of an OD600-dependent ΔbipA 18° ribosome profile defect …… 87

Figure 2.7 Uup and EF-G suppress Δ*bipA* rifampicin sensitivity 88

Figure 2.8 Translation fidelity in a Δ*bipA* mutant …….. 89

Figure 2.9 Translation fidelity in a Δ*uup* mutant …….. 90

Figure 2.10 Deletion of Uup results in decreased polysomes ……. 91

Figure 2.11 Uup and EttA are similar …….. 92

Figure 2.12 While mutants of uup and ettA have different phenotypes, high-copy Uup and EttA suppress each other's ribosome profile defects…….. 93

Figure 2.13 Overexpression, but not loss, of Uup reduces viability…….. 94

Figure 2.14 Uup may be important for translation of *topA* ……. 95

Figure 2.15 Uup may also be an E-site translation factor…… 96

Figure 3.1 Effects of loss of ribosomal protein L1 on P-site and A-site fidelity …….. 132

Figure 3.2 Loss of the L1 protein results in an accumulation of 50S ribosomal subunits …… 133

Figure 3.3 High copy suppressors of ΔL1 cold sensitivity ……. 134

Figure 3.4 Relationship of RpoS-related high-copy suppressing proteins of ΔL1 slow growth in the cold …… 138

Figure 3.5 The ribosome-binding/affecting sites of ribosome-related suppressing proteins clusters around the PTC …… 139

Figure 3.6 Not all the high-copy suppressors of ΔL1 slow growth also suppress the ΔL1 ribosome profile defect …….. 140

Abstract

The ribosome is comprised of two subunits, formed from three ribosomal RNAs and many ribosomal proteins. The two subunits come together around a messenger RNA during initiation, and catalyze the addition of sequential amino acids to a growing polypeptide chain as directed by the messenger RNA during elongation. While much of the ribosome is a relatively rigid molecular machine, there are two flexible protrusions comprised of rRNA and ribosomal proteins. One of these protrusions is the L1 arm where deacylated tRNAs leave the ribosome. The contributions of the L1 stalk and associating factors are unclear. In this work, I report that loss of the protein L1 results in changes in translation accuracy and perturbation of the ribosome profile. I also find that proteins involved in RpoS regulation/regulon, proteins affecting the structure of the ribosome in the vicinity of the active site, and proteins affecting DNA replication are able to suppress, when at high copy, the previously-reported slow growth the of Δ*rplA* (encoding L1) mutant. Recent studies have revealed that translation factors act on the L1 arm side of the ribosome. I have characterized Uup, an *E. coli* ABCF protein that shares many of the residues involved in binding to tRNAfMet and ribosomal protein L1 in the closely related *E. coli* ABCF protein EttA. Exogenous Uup suppresses many phenotypes

caused by deletion of the elongation family GTPase BipA, including a growth-dependent ribosome profile defect. Both translation of reporter plasmids and translating ribosomes are reduced in *Δuup*. While *Δuup* and *ΔettA* show different phenotypes in multiple assays, each can suppress defects resulting from loss of the other. I propose that Uup, as shown for EttA, can promote formation of the first peptide bond in the elongation phase of translation. This adds to the short but rapidly growing list of translation factors regulating translation at the E-site.

Chapter 1

Introduction

THE BACTERIAL RIBOSOME

The bacterial ribosome consists of enzymatically-active ribosomal RNA (rRNA) and many associated proteins. The ribosome initially assembles as two subunits, the large, or 50S subunit and the small, or 30S subunit. The large subunit is comprised of two rRNAs (23S and 5S) and ~33 ribosomal proteins, depending on assay conditions [1]. The small subunit is comprised of one rRNA (the 16SrRNA) and 21 proteins [1].

The small (30S) ribosomal subunit is the first subunit to interact with the messenger RNA (mRNA) and initiator tRNA during the initiation of translation [2]. Interaction of the mRNA with the small subunit establishes the reading frame for translation [2, 3]. The small subunit has three sites where the anti-codon loop of transfer RNA (tRNA) bringing amino acids to the ribosome during translation interact more or less strongly with the mRNA codons and the 16S rRNA [4-6]. These are called the A, P, and E sites. The A (aminoacyl) site is the site where tRNAs first enter the

ribosome [7]. The P (peptidyl) site is nearer the center of the ribosome [8, 9], and the E (exit) site, from which deacylated tRNAs exit the ribosome, is on the opposite side of the ribosome from the A site [5, 6].

The 50S subunit contains equivalent A, P, and E sites, but interacts with the amino-acyl ends of tRNAs [10]. The peptidyl transferase activity of the ribosome, which incorporates new amino acids into the growing peptide chain, is a feature of the 50S P-site [11, 12]. The 50S subunit A, P, and E sites make weaker interactions with the tRNAs than the codon/anticodon mediated 30S connections, allowing the amino acid-binding ends of tRNAs bound to the ribosome to vacillate between 50S sites without changing their 30S site occupancy [13]. This vacillation allows the same tRNA to simultaneously occupy different sites in the 30S and 50S subunits, in what is termed a hybrid state [14].

The two subunits along with an mRNA and an initiator tRNA bearing an fMet amino acid bound on the mRNA start codon in the P-site of the ribosome form the 70S holoribosome [15, 16]. Incoming amino-acylated tRNAs are delivered to the A site of the ribosome by EF-Tu [17, 18]. After a peptide bond is formed between the amino acid(s) attached to the P-site tRNA and the amino acid on the newly arrived tRNA, the peptide chain is transferred to the A-site tRNA [19, 20]. The A and P site tRNAs are translocated to the P and E sites by the action of EF-G [18, 21].

While most of the ribosome is a tightly-packed mass of proteins and rRNAs, there are two protuberences from the 50S subunit that are quite flexible (Fig. 1.1). The larger of these protuberences is called the L7/L12 stalk, because it is comprised primarily of the large ribosomal subunit protein L7/L12 [22]. The L7/L12 stalk is located on the large ribosomal subunit near the A-site side of the ribosome, where charged tRNAs dock on the ribosome [22-24]. The proteins L10 and L11 are located along the L7/L12 stalk towards the central mass of the large subunit [25-28].

The other protuberence of the 50S ribosomal subunit is found on the opposite side of the ribosome from the L12 stalk, near the E-site, where deacylated tRNAs exit the ribosome [29]. It is called the L1 stalk, and is comprised of flexible rRNA bound by ribosomal protein L1 [29-31].

Ribosomal protein L1

The 23S rRNA is divided into six regions, or domains, that fold into units of secondary structure. The L1 protein binds the ribosome at a region in domain V of the 23S rRNA between bases 2105 and 2184 (Fig. 1.1, inset) [30, 32]. This region contains a loop at the intersection of two perpendicular helixes (helixes 76 and 77), as well as two other loops. The intersection between helixes 76 and 77 contains two conserved G-C base pairs and a conserved A-G interaction that are necessary for recognition by L1 [30, 33]. L1 binds directly to the rRNA, and is predicted not to be important for the binding of any other ribosomal protein [34].

The protein L1 is the largest ribosomal protein, containing 233 amino acids. L1 has the high Lys/Arg ratio characteristic of ribosomal proteins and, with about 70% of ribosomal proteins, is conserved in all three domains [35-38]. Structurally, the amino acid sequence of L1 folds into two domains. The larger domain, domain I, is interrupted by the sequence of the smaller domain II [30]. Both domains form globular structures that bend at the "hinge" region that connects them. The interface of the two domains forms the rRNA binding region of L1, and the accessibility of this region strongly influences the strength of L1-RNA binding [30]. Domain I of L1 makes the majority of contacts with the rRNA [30, 31, 33] and interacts primarily with helix 77 of the L1 arm, including the conserved residues of the binding site on the rRNA [30, 31]. Domain II makes fewer contacts with the rRNA, primarily in the interconnecting loop region between nucleotides G2165 [30, 31, 33].

rplA mutants were initially obtained as spontaneous revertants of kasugamycin and spectinomycin dependant mutants. These *rplA* mutants grew slowly and showed decreased protein synthesis and a defect in tRNA binding [39-41]. The tRNA binding defect was specific to initial amino-acylated tRNA binding in the P-site [42]. It was concluded that the reduction in protein synthesis resulted from a decrease in the ribosome-stimulated GTPase activity of EF-G on these *rplA* mutants [41].

L1 appears to be a regulator of E-site occupancy [10, 43]. The flexible L1 arm is ideally placed to contact exiting tRNAs and either block their exit from the ribosome

or stabilize their ribosomal interactions. More recent work has shown that L1 and the L1 arm can make direct contact with the elbow region of both E and P/E-site tRNA [44-48]. The L1 arm moves in a coordinated fashion during the motion of the ribosome that accompanies translocation, suggesting that L1 may help guide the tRNA during its P to E-site transition [48]. Contact between L1/the L1 arm and the P-site tRNA is made immediately after petidyl transfer, and repeated cycles of association/dissociation between L1/the L1 arm and the P-site tRNA accompany the spontaneous P-E/P transitions of the tRNA and coordinated formation of the racheted/unracheted states of the ribosome [48].

Elongation Factor G (EF-G) is a GTPase that catalyzes translocation of tRNAs from the A and P sites to the P and E-sites [10, 18, 21]. EF-G binding to the ribosome shifts the equilibrium of the L1 arm in a pre-translocation ribosome towards a "closed" position and shifts the equilibrium of ribosomal states towards the racheted state [48, 49]. This shift promotes sustained contact between L1/the L1 arm and the P/E tRNA. This contact between L1 and the tRNA is maintained throughout the process of translocation and is important for efficient translocation [44, 48, 50-53]. The role of L1 in effective translocation may explain the defective interaction between EF-G and the ribosome during protein synthesis previously seen in mutants lacking L1 [41].

L1/the L1 stalk interacts differently with initiator tRNA than with tRNAs usually involved in elongation. The initiator tRNA translocates more slowly than elongator

tRNAs and spends more time in the classical P/P state than in the hybrid P/E state (which involves L1-tRNA contacts) prior to translocation [14, 49, 54-56]. The L1/L1 stalk interactions with P-site tRNA also are broken more quickly when a $tRNA^{fMet}$ occupies the P-site than when other tRNAs occupy the site, and the L1 stalk spends increased time in the open position on ribosomes with a $tRNA^{fMet}$ in the P-site compared to ribosomes containing elongator tRNAs in the P-site [49]. Computer models based on crystal structures predict the $tRNA^{fMet}$ sits deeper into the P-site than elongator tRNAs, and thus makes fewer contacts with L1 and the L1 arm, explaining the slower translocation of the initiator tRNA [47].

A number of proteins have been shown or proposed to interact with L1 as part of their activity on the ribosome. In yeast, the essential protein eEF3 is proposed to modulate the position of the L1 arm and regulate E-site occupancy during translation [57-59]. In bacteria, proteins EF-P and EttA contact both the P-site tRNA and L1/the L1 arm [60-62]. EF-P promotes peptide bond formation between amino acids that are poor donors and/or acceptors in the peptidyl transferase reaction [63]. EttA binds certain characteristics unique to the initiator $tRNA^{fMet}$, and is able to either inhibit or promote formation of the first peptide bond of protein synthesis, depending on the level of cellular energy [61, 62].

The gene encoding *rplA* (L1 gene) is the second gene in a small, two-gene ribosomal protein operon. The first gene in the operon encodes ribosomal protein L11. Both genes are primarily transcribed from the promoter region located at the beginning

of the gene for L11 (*rplK*), although there could also be some initiation from a potential promoter region predicted at end of *rplK*. The L11-L1 operon is also translationally controlled by feedback inhibition mediated by L1 [64, 65]. L1 not bound to the ribosome binds to an mRNA structure similar to its ribosomal binding site located at the beginning of the L1-L11 mRNA, preventing translation of both genes [30, 33, 66-68]. The interaction of L1 with the mRNA is much weaker than the interaction with the rRNA, allowing swift de-repression of translation in the presence of free ribosomal RNA [30, 33]. There is an increased level of L11 protein detectable in *rplA* mutants, demonstrating that the L11 over-produced in the absence of L1 control is stable *in vivo* [69, 70].

Ribosomal Protein L11

L11 also binds directly to the rRNA [27]. It makes weak contacts with L7/12, L5, and L15 [34]. L11 has been shown to bind to the assembled ribosome at the base of the L7/12 stalk (Fig. 1.1) [26-28]. Its C-terminal domain (CTD) associates tightly with a region of the ribosome where the translational GTPases also interact [28]. Its N-terminal domain (NTD) is less structured, and able to shift its conformation [28]. The N-terminal domain is located closer to the sarcin/ricin loop [28]. Based on these location and conformational characteristics, it was proposed that L11 could be involved in modulating the binding or activity of elongation family GTPases, such as EF-G and BipA [27].

This supposition is supported by cry-electron microscopy visualizations of the ribosome with bound EF-G. EF-G has been shown to make ribosomal contacts near the location of L11 on the ribosome [71, 72]. After GTP hydrolysis, L11 participates in formation of an "arc-like connection" (ALC) where the tip of the NTD of L11 appears to move towards the G' domain of EF-G [28]. Meanwhile, domain V of EF-G moves away from the G' domain into a cleft between the 23S rRNA and the NTD of L11 [28]. The rearrangement of EF-G described here could be important for allowing domain IV of EF-G to make the appropriate contacts to promote translocation [28]. The ALC is also formed during binding of EF-Tu to the ribosome, making it probable that L11 also interacts with that GTPase [73]. The combination and coordinate expression of E-site (L1) and A-site (L11) ribosomal proteins in the same small operon may reflect the coordination or "cross-talk," between the A and E sites of the ribosome.

TRANSLATION

Initiation

The process of translation is comprised of three distinct steps, termed initiation, elongation, and termination. During initiation, the elongation-competent 70S ribosome is formed from the ribosomal subunits, mRNA, and initiator tRNA (Fig. 1.2). Three proteins, termed initiation factor (IF) 1, 2, and 3 are important to this process. Initiation begins with the formation of an initiation complex on the 30S subunit. FRET studies indicate that IF3 and IF2 bind to the 30S early in the formation of the initiation complex and on a similar time scale [2]. IF1 binding

occurs on a longer time scale than the other IFs, but is not necessarily dependent on their binding [2, 74]. The mRNA is able to bind to the 30S subunit independently of the IFs [2, 74]. After mRNA binding, IF2 mediates binding of the initiator $tRNA^{fMet}$ at the start codon in the P-site of the 30S subunit [3]. Thus, initiation on the 30S subunit is critical for accurately setting the reading frame for the mRNA. After assembly of the 30S initiation complex, the 50S subunit is able to bind to the programed 30S ribosome, and the IFs dissociate, leaving a 70S ribosome with a P-site $tRNA^{fMet}$ and an empty A site, ready to enter elongation (for review see [75, 76]).

IF1 is an essential initiation factor with homologs in all domains of life [77-79]. Some mutants of *infA* (encoding IF1) result in increased translation of mRNAs with longer leader sequences, leading researchers to propose that IF1 may have a dampening effect on translation of such mRNAs [80]. Sub-lethal doses of kasugamycin are able to produce the same phenotype in wild type cells, and to enhance the phenotype in *if1* mutants [80]. Mutations in *yggJ*, *bipA*, or *cspA* suppress the cold-sensitive if1 mutant phenotype [80].

IF2 is a GTPase protein that binds on a well-characterized G-protein binding site shared by the elongation GTPases (discussed later) on the A-site side of the ribosome. The IF1 binding site may be partially occluded by IF2, and IF2 occupancy of the ribosome impedes IF1 binding [2, 81]. IF2 makes contact with the incoming $tRNA^{fMet}$ and promotes binding of the initiator tRNA to the ribosome to the exclusion of other tRNAs [3, 82]. The presence of both the initiator tRNA and IF1

promotes continued occupancy of the initiation complex by IF2 [2]. IF2 promotes association of the 30S initiation complex with the 50S subunit, most likely via its interactions with the GTPase associated region (including L11) and the 30S-interface region of the 50S subunit [83-87]. Subunit joining results in hydrolysis of IF2-bound GTP and dissociation of IF2 from the 70S complex [88].

IF3 is important for the accuracy of initiation. IF3 interferes with 50S association with 30S complexes carrying non-AUG start codons and non-fMet tRNA in the P-site [15, 89, 90]. IF3 binding can sterically occlude key inter-subunit bridge contacts, providing a structural rationale for its regulation of 50S binding [81, 91]. Whether IF3 dissociation from the 30S initiation complex is necessary for subunit joining is debated, but IF3 is very flexible, and has been shown to adopt multiple conformations on the 30S initiation complex [15, 92, 93]. Therefore, subunit joining could be accomplished while IF3 is still present on the 30S subunit.

Initial binding of the mRNA to the 30S complex is based on interaction of its ribosome binding site with the 30S ribosome [94, 95]. Highly structured mRNAs bind less efficiently than less-structured mRNAs and may therefore be translated less efficiently[96]. Binding of the $tRNA^{fMet}$ anti-codon loop to the start codon of the 30S-bound mRNA triggers a conformational change that allows 50S binding to the 30S complex [97]. Another conformational change after binding of the 50S subunit confirms correct tRNA binding in the P-site, and produces elongation-ready 70S ribosomes [2, 83].

Elongation

Elongation is the primary, repetitive stage of translation. During elongation, GTPases EF-Tu and EF-G catalyze the sequential addition of amino acids encoded by the mRNA into the growing polypeptide chain.

Ribosomes with peptidyl tRNA in the A-site fluctuate between hybrid and classical states [13]. EF-G binding shifts the equilibrium between these states towards the hybrid state [13, 55, 98]. In the hybrid state, the L1 arm is tucked in towards the tRNA, an interaction that may contribute to tRNA movement during translocation [47, 48, 53, 55, 99-101]. The 30S subunit is also rotated relative to its usual position with respect to the 50S subunit. Re-arrangements of the ribosome and of the EF-G domains (in a process loosely coordinated by GTP hydrolysis) produce rapid and uni-directional translocation of tRNAs from the 30S A and P sites to the 30S P and E sites (Fig. 1.3). EF-G also helps prevent reversal of the tRNA motion, while 16S rRNA loops prevent backward slippage of the mRNA [13, 102-104].

Once the A-site has been emptied by translocation, EF-Tu brings the appropriate charged tRNA to the A-site of the ribosome [7, 73]. The peptidyl transferase activity of the 50S subunit catalyzes the formation of a peptide bond between the amino acid on the A-site tRNA and the peptide chain on the P-site tRNA [19, 20]. The process continues with cycles of translocation and addition of tRNAs until a stop signal is reached.

Not all peptide bonds are as easy to make as others, and a number of proteins have been proposed to promote formation of peptide bonds under specific circumstances. EF-P is an elongation factor that promotes formation of peptide bonds on stall-prone amino acid sequences, primarily involving multiple encoded prolines [63, 105, 106]. EttA is an ATPase recently shown to bind in the E-site of the ribosome and promotes or stalls the formation of the first peptide bond, involving the fMet, depending on cellular energy levels [61, 62]. All the elongation factors mentioned in this section will be discussed in more depth later in this document.

Termination/Recycling

After translation of a complete message, the amino acid chain must be freed from the ribosome, and the ribosomal subunits must be freed to reassemble at a new start codon. Termination of translation is signaled by a non-coding, or "stop" codon. In *E. coli*, there are three stop codons: UGA, UAG, and UAA [107]. The base after a stop codon also contributes to the strength of the stop codon [108]. Stop codons are not recognized by tRNAs, but are instead recognized by class 1 release factors (RFs). There are two class 1 RFs, RF1 and RF2, both of which recognize and terminate transcription at UAA codons [109-111]. RF1 also recognizes and terminates transcription at UAG, and RF2 also recognizes and terminates transcription at UGA [109-111].

RF1 or 2 binds in the A-site of a ribosome programed with an appropriate stop codon (Fig. 1.4) [112-114]. They contain features that are able to interact with the stop codons and the peptidyl transferase center. Tripeptide motifs PxT in RF1 and SPF in RF2 interact with and decode the stop codons [115]. Both RF1 and RF2 also contain a conserved GGQ motif that interacts with the peptidyl transferase center and is required for peptide release function [112-114, 116-120]. RF2 is about 5 times more abundant than RF1 in exponentially growing cells [121-123].

Stop codon usage in genes expressed in exponentially growing cells reflects both the GC content of the organism and the overlap of the release factors [123]. As GC content increases, the use of UGA increases and the use of UAA decreases [123]. However, UAG levels do not change significantly between organisms with different GC levels[123]. UAA, which is recognized by both RF1 and 2, is most commonly used as the termination signal for mRNAs that are expressed highly, while UAG, which is more prone to read-through, is predominantly used to terminate messages with lower expression levels [123, 124].

RF3, the bacterial class 2 RF, is a GTPase that promotes dissociation of RFs 1 and 2 from the ribosome, freeing them for successive termination events [125, 126]. RF3 is not essential in *E.* coli, and is absent in many bacteria [127]. Some research has indicated a preference of RF3 for recycling RF2, but other data shows no preference [126, 128]. RF3 is most effective at dissociating RF1 and 2 after the amino acid chain has been released from the P-site tRNA, supporting the view that dissociation

of these RFs may be tied to a steric clash predicted to occur as a consequence of ribosome rotation [127, 129].

Dissociation of the 70S ribosome after release of the translated protein seems to also result from intersubunit rotation [130]. Ribosome recycling factor (RRF) initially interacts with helices 43 and 95 of 23S rRNA and the L11 region at the base of the L7/12 stalk [130]. EF-G binding to the ribosome complexed with RRF drives both the P-site tRNA and RRF towards the E-site while bringing the ribosome into an unratcheted state [130]. These motions are predicted to disassemble the 70S ribosome to allow reinitiation elsewhere [130]. IF3 also acts in ribosome recycling after termination. IF3 binding helps prevent premature reassembly of the subunits [131, 132].

ELONGATION FACTORS

GTPase Elongation Protein Family

There are four *E. coli* proteins with related structures known as the GTPase elongation protein family (Fig. 1.5) [133, 134]. These proteins, EF-G, EF-Tu, LepA, and BipA, share up to four N-terminal domains, two of which are involved in ribosome binding and GTP hydrolysis [133]. Their C-terminal domains are unique, and presumably mediate their unique functions. This family includes the conserved, essential elongation proteins EF-Tu and EF-G, as well as the non-essential and less conserved LepA and BipA proteins [133]. While EF-Tu and EF-G functions are well established, the activities of LepA and BipA are only beginning to be elucidated.

EF-Tu

EF-Tu concentration in the cell is equivalent to the concentration of tRNA, and higher than the concentration of ribosomes [135]. EF-Tu brings a charged tRNA to the A-site of the ribosome at the beginning of each round of elongation [73]. After initial cognate tRNA binding to the ribosome, the bound GTP is hydrolyzed and EF-Tu leaves the ribosome [136, 137]. The guanine exchange factor Ts facilitates the exchange of bound GDP for GTP, EF-Tu binds charged tRNA, and the cycle continues [138]. The traditional view has been that once nucleotide exchange occurs, Ts releases EF-Tu*GTP to find a charged tRNA partner [7, 139-144]. Recent findings indicate that Ts most likely remains bound to the EF-Tu*GTP complex and facilitates tRNA binding by lowering the activation energy of the tRNA binding intermediate [138, 145].

In order to facilitate encoded tRNA binding in the A-site of the ribosome, EF-Tu must be able to bind and release a wide range of charged tRNAs. The affinity of tRNAs for EF-Tu is carefully balanced, and changes in affinity of EF-Tu for tRNA alter the elongation rate in *E. coli* [146]. The majority of the affinity of EF-Tu for charged tRNA is mediated by two regions of EF-Tu [147]. The first region is the amino acid binding pocket of EF-Tu, where the amino acid on the charged tRNA can make varied contacts with EF-Tu depending on its size and charge [148, 149]. The second region that contributes significantly to the strength of tRNA binding is a sequence of three conserved amino acids on EF-Tu that interact with the T-stem of tRNA. There is extremely high sequence variability in the three tRNA residues participating in

this interaction [146]. Also, the strength of the amino acid-mediated interaction must vary depending on the nature of the amino acid [150]. However, elongator tRNAs bind EF-Tu with very similar affinities, indicating that differences in binding affinity at one of these locations is compensated for by differences in affinity at the other [146, 150-153].

EF-Tu binds to the ribosome at a binding site overlapping that of the other elongation family GTPases [18, 144]. This includes contact with the sarcin-ricin loop and the L11 region. Contact with the sarcin-ricin loop helps maintain EF-Tu binding to the ribosome during tRNA docking and GTP hydrolysis, but is less important for EF-Tu than for the structurally related EF-G [154-156]. Contacts with helixes 42-44 of the 23SrRNA along with ribosomal proteins L7/L12, L10 and L11 are also made upon EF-Tu docking on the ribosome [157-159].

EF-Tu delivery of aa-tRNA proceeds via a multi-step process that produces the high level of accuracy seen in translation. Initial docking of the EF-Tu*GTP*aatRNA ternary complex is followed by a codon recognition step in which the anti-codon loop of the bound tRNA enters the A-site on the 30S subunit and interacts with the mRNA codon in the A-site [7, 139, 160, 161]. A match between the codon in the A-site and the anti-codon loop triggers a conformational change that is transmitted to EF-Tu and results in GTP hydrolysis [139, 160-162]. Interaction of non-cognate tRNAs does not result in GTP hydrolysis on the time scale of the non-cognate ternary complex association with the ribosome, and near cognate binding produces

only slow hydrolysis of GTP resulting in low incorporation [139, 160, 162]. The precise mechanism for transmission of cognate tRNA binding signal resulting in GTPase activation is debated, and equivalent data have been interpreted as support for significantly varying models [137, 157, 163]. However, the complete tRNA is necessary for GTP hydrolysis to result from cognate tRNA binding, implicating tRNA in transmission of the hydrolysis signal [137, 157, 163, 164]. Histidine 84 is essential for the GTPase activity of EF-Tu, and L7/L12 contacts also make important contributions to GTPase activity [165-167].

Following GTP hydrolysis, the EF-Tu-GDP complex adopts a conformation with low affinity for tRNA and the ribosome, and dissociates [168]. Dissociation of EF-Tu frees the tRNA to fully interact with the A-site codon[10, 168, 169]. Cognate tRNA accommodates into the A-site on the 30S subunit, while near-cognate and non-cognate tRNA is more likely to diffuse away [170, 171]. 30S A-site accommodation of the anti-codon loop allows the remaining regions of the tRNA to settle into the 50S A-site and interact with the peptidyl transferase center [10, 171]. Accommodation provides a second kinetic proofreading step, increasing the accuracy of translation [170, 171].

Control of EF-Tu is an important mechanism for cellular control of translation. The guanine exchange factor, Ts, which helps EF-Tu exchange GDP for GTP after ribosome-stimulated hydrolysis, also mediates turnover of the GTP bound in the EF-Tu*GTP*tRNA ternary complex [138, 145]. This provides a mechanism for the rapid

cessation of translation upon depletion of cellular energy by reincorporation of GDP instead of GTP and consequent dissociation of the ternary complex [138]. In another example of control of translation via control of EF-Tu, Hsp33 mediates rescue of the cellular effects of excess misfolded protein in *tig dnaK* mutants by facilitating the degradation of EF-Tu and thereby reducing protein synthesis [172].

EF-G

EF-G binds to the ribosome at a binding site on the A-site side of the ribosome overlapping that of the other elongation family GTPases, again including the sarcin-ricin loop and the L11 region [18, 154]. The sarcin-ricin loop plays a much stronger role in EF-G binding to the ribosome than in EF-Tu binding [155, 156]. Domain V of EF-G interacts with the L11 region at the base of the L7/12 stalk, and also makes significant contributions to ribosome binding [71, 173]. Domains III, IV, and V create a structural mimic to the tRNA bound to EF-Tu [71, 174]. Domain IV interacts with the A-site tRNA in the pre-translocation ribosome and maintains that contact as the peptidyl tRNA is translocated to the P-site [71, 174, 175]. A conserved histidine likely mediates GTP hydrolysis in EF-G similarly to in EF-Tu[136, 166]. Ribosomal protein L7/L12 is extremely important for phosphate release after GTP hydrolysis by EF-G, and L7/12 mutants can stall EF-G on the ribosome [23, 24].

The pre-translocation ribosome spontaneously fluctuates between a “classical” state, where each tRNA occupies the same site on both the 30S and 50S subunit, and a “hybrid” state, where the anti-codon loop of the tRNA is seated in its pre-

translocation site on the 30S subunit, but the acceptor stem has moved toward or into its post-translocation site on the 50S ribosome [21, 55, 98, 176, 177]. During formation of the hybrid state, the 30S ribosomal subunit rotates relative to the 50S subunit, most likely facilitating the tRNA occupation of hybrid binding sites (see Fig. 1.3) [21, 51, 56, 178-180]. It had previously been thought that EF-G bound preferentially to hybrid state ribosomes [181], but more recent work demonstrates that EF-G binds to both ribosome states equivalently and shifts the ribosome equilibrium towards the hybrid state upon binding [13, 182, 183].

Ribosome binding by EF-G is followed by domain reorganization and GTP hydrolysis [183, 184]. Both domain rearrangement and GTP hydrolysis are essential for EF-G mediated translocation [184, 185]. However, GTP hydrolysis and EF-G rearrangement do not seem to be strictly coupled [186, 187]. While the domain configuration of EF-G in solution tends towards a compact form, EF-G primarily adopts an extended conformation in which domain 4 is extended away from the rest of the protein towards the ribosomal A-site when bound to the ribosome [186, 188]. This extension is most pronounced in post-translocation ribosomes, where domain IV of EF-G blocks the A-site and makes contact with the peptidyl tRNA in the P-site [175]. The change in EF-G conformations on pre and post translocation ribosomes is accomplished by a rotation of EF-G around the sarcin-ricin loop, which maintains its position throughout translocation, and by rearrangement of EF-G domains III, IV, and V in relationship to domains I and II [175, 188].

The data suggest that a further movement of the "head" of the 30S subunit relative to the body of the 30S subunit "unlocks" the ribosome, opening a channel and permitting rapid movement of the mRNA and associated tRNA [13, 100, 189-191]. Return movement of the 30S body and head finishes translocation of the mRNA and associated anti-codon loops, and presents a ribosome with tRNAs in the E and P site and an empty A site [175, 190-192]. EF-G likely contributes to rapid translocation both by reducing the energy difference between intermediate states [13, 193] and by preventing the ribosome from returning to previous states, providing directionality to an otherwise reversible process [101, 193-197].

It is unknown whether the GTPase activity of EF-G is triggered on binding to the ribosome, or after translocation has begun [184]. GTPase activity is required for completed translocation, but not for EF-G docking to the ribosome or for formation of intermediate states, suggesting hydrolysis may be tied to the final steps of translocation and reverse movement of the 30S subunit [102-104, 184, 190].

LepA

LepA is another member of the translational GTPase family and shares domains I, II, III, and V with EF-G [198, 199]. Unlike EF-G and EF-Tu, the codon usage of LepA suggests that it is expressed to a low degree, a suggestion borne out by direct studies [198, 200]. *lepA* deletion mutant shows little phenotype under the optimal growth conditions commonly used in the laboratory, frustrating early characterization attempts [201]. However, this protein is extremely conserved in

bacteria and in bacterial-derived organelles [202] and is highly toxic when over-expressed [199]. More recent work showed LepA is important for *Helicobacter pylori* survival of high-acid conditions [203] and for normal levels of protein synthesis under stress conditions including low temperature, high ionic, and acidic conditions in *E. coli* [202, 204]. Yeast mitochondria also require LepA (Guf1) for normal function under temperature and nutrient stress, and mitochondrial translation is decreased in the absence of functional mitochondrial LepA [205].

LepA lacks domain IV of EF-G, which has been proposed to prevent back-translocation during EF-G interaction with the ribosome [206]. Instead, LepA contains a unique C-terminal domain that can interact with the A-site tRNA [206]. A crystal structure of the ribosome bound to LepA shows this C-terminal domain reaching through the A-site and interacting with the P-site tRNA [207]. The 30S subunit of LepA-bound ribosomes shows a reverse ratcheting from that seen in EF-Tu bound structures [207].

LepA has been shown to back-translocate the tRNA-mRNA complex in the ribosome, effectively reversing the activity of EF-G [199, 206, 208]. Early experiments showed that addition of LepA can increase the proportion of functional protein produced in an *in vitro* expression system, prompting the hypothesis that LepA reverses translocation of incorrect tRNAs, permitting the ribosome a second chance at accurate translation [199]. However, LepA was not able to reverse the effect of error-prone antibiotics on translation fidelity in *in vitro* systems [199]. Later work

showed a similar effect, where deletion of the mitochondrial paralog of *lepA*, *guf1*, resulted in apparently wild type levels of overall protein production in the mutant under temperature stress, but high levels of insoluble protein [205]. Seven proteins were detected at lower levels in Δ*lepA* cells grown at acidic pH. Although no ribosomal proteins or assembly factors were found among the seven proteins specifically under-expressed, an OD_{600}-dependent 30S ribosomal subunit assembly defect was observed in the mutant [204]. This may be the result of under-expression of a number of proteins involved in tRNA and amino acid biosynthesis [204].

The inability of researchers to find translation fidelity defects in a Δ*lepA* strain has led to the suggestion that the ability of LepA to promote production of function protein is due to increasing the soluble fraction of proteins, possibly by slowing translation by either back-translocating the ribosome or by competition with EF-G [199, 209, 210]. However, other researchers find that addition of LepA to an *in vitro* system increases amino acid incorporation, and therefore propose that LepA directly increases translation by "un-pausing" ribosomes that become stuck during elongation under extreme conditions [202].

BipA

BipA is the last member of the elongation GTPase family [211]. It contains the ribosome binding domains characteristic of this family, and is expected to bind to the ribosome at the same location involving the sarcin-ricin loop and the L11 region [212]. BipA is widely conserved in bacteria, but is not as pervasively distributed as

LepA [211]. Like LepA, BipA is not essential under normal laboratory condition, but becomes important under stress [213-216]. BipA has also been shown to be important for pathogenicity in pathogenic *E. coli* strains[214, 217].

BipA is important for growth at low temperatures and under SDS stress in pathogenic and non-pathogenic bacteria[216, 218] and has been shown to affect the production of both pathogenicity determinants and housekeeping genes, most likely by translational control of an unidentified central regulator protein [214, 215, 217, 219, 220]. Although our unpublished work shows BipA present across ribosomal subunits and translating ribosomes (see chapter 2), BipA has been shown to associate primarily with the 70S ribosome under optimal growth conditions but to change its primary association to the 30S subunit under stressed conditions [221].

The 70S association of BipA is dependent on GTP, but the 30S binding requires increased ppGpp levels [221]. Addition of low levels of ppGpp to the reaction mix increased the GTPase activity of BipA, but continued addition of ppGpp reduced the ribosome-stimulated GTPase activity [221]. These results are consistent with the view that BipA regulates translational response to stress conditions [221]. BipA control of virulence factors may have developed as a result of stress conditions accompanying initiation of infection.

The cellular requirement for BipA is dependent on ribosome modification by RluC. RluC pseudouridylates nucleotides 955, 2504, and 2580 of the 23S rRNA[222].

Pseudouridylation at two or more of these nucleotides is required for the cold-sensitivity of Δ*bipA* strains [133]. Deletion of *bipA* results in a 50S ribosome biogenesis defect that may be secondary to a translation defect [213]. Deletion of *rluC,* relieves both the cold-sensitive slow growth and the ribosome assembly defect of a *bipA* mutant [213]. Deletion of *rluC* also suppresses a Δ*bipA* capsule biosynthesis defect by increasing synthesis of capsule proteins [133].

The Δ*bipA* phenotype also has genetic interactions with mutants lacking DeaD, a ribosome assembly factor, and IF1, a non-essential initiation factor that seems to retard initiation on messages with long leader sequences [80, 213]. The Δ*bipA*Δ*deaD* double mutant shows synthetic defects in both growth and ribosome assembly in the cold [213]. Deletion of *bipA* suppresses the cold-sensitivity of mutants of IF1 that cause excess translation of mRNAs with longer leader sequences, leading these researchers also to suggest that the role of BipA may be to promote translation [80].

Non-GTPase Bacterial Elongation Factors

EF-P

EF-P is a universally conserved bacterial elongation factor [78]. In bacteria, EF-P is not essential, but mutants grow very slowly [223]. Homologs in archaea and eukarya are essential [78].

EF-P was originally identified as an initiation factor, because it increases the puromomycin reactivity of initiating ribosomes *in vitro* [224, 225]. However, EF-P

does not affect the production of fMet-Lys or fMet-Phe dipeptide in *in vitro* systems [226]. EF-P does promote the production of poly-P tripeptides, but not most other tri-peptide sequences (Fig. 1.6A) [63, 106, 226]. *In vivo*, *efp* mutants show ribosome stalling and decreased production of certain proteins containing PPP runs and certain other stall-prone sequences [63, 105, 106, 227-230]. Plasmid-encoded EF-P relieves stalling on these sequences [106]. These data have led to the conclusion that EF-P primarily acts on the ribosome to promote peptide bond formation in the face of amino acid sequences particularly resistant to peptide bond formation, such as the PPP sequence [63, 106, 226, 231-235].

EF-P folds into a conformation similar to a tRNA and binds to the ribosome at a site between the E and P sites, and overlapping with the E-site [60, 236, 237]. EF-P consists of three structural domains [60]. Domain I contacts the P-site tRNA near the anti-codon stem loop on the 30S subunit [60]. Domain III is positioned to interact with the P-site tRNA acceptor stem, and extends into the E-site [60]. The L1 arm is drawn in towards the EF-P-bound ribosome, and domain III of EF-P binds between domains I and II of L1 [60].

In *E. coli*, EF-P is postranslationally lysinylated by EmpA, and B [237-239]. This modification is necessary for function, and loss of these enzymes produces a phenotype comparable to *efp* mutants [63, 106, 238, 240]. EF-P is also hydroxylated by EmpC, but this modification seems to have little effect on function [106, 226, 239, 240].

A wide range of proteins are regulated by EF-P, explaining the diversity of phenotypes seen in *efp* mutants, including motility defects, membrane defects, and ribosome profile abnormalities [226, 227]. The only universally conserved PPP motif is essential to the proofreading function of valine tRNA synthase [241]. The vital function of the PPP sequence (which requires EF-P intervention for efficient translation) in this important protein may be the primary evolutionary pressure leading to the conservation of EF-P and its homologs [241].

EttA

EttA (YjjK) is one of four *E. coli* ATPases of the ABCF (ATP binding cassette F) subfamily [242]. The ABCF subfamily is comprised of atypical ABC proteins that lack the trans-membrane domains found in the majority of ABC proteins (for reviews, see [243-247]). While the functions of the *E. coli* ABCF proteins are just beginning to be elucidated, ABCF members with known functions in other organisms are involved in translation and/or ribosome biogenesis. For instance, the *S. cerevisiae* ABCF member, eEF3, is an essential elongation factor that regulates E-site occupancy [59, 248]. ABC50, a human ABCF protein, interacts with eIF2 to promote accurate start-sight selection on initiating ribosomes [249-252].

The four ABCF proteins in *E. coli* are EttA, Uup, YheS, and YbiT [242]. All have been proposed to affect ribosome function, but only EttA has been shown to interact with

the ribosome [61, 62, 253]. EttA is not essential at any temperature or nutrient-availability condition tested [254].

EttA is predicted to be highly expressed in many bacteria and is the only bacterial protein of the ABCF family for which a function is known [255, 256]. EttA has recently been shown by cryo-EM to bind in the E-site of the ribosome and contact both the L1 stalk and the P-site tRNA (Fig. 1.6B) [62]. EttA interacts with distinctive features of the initiator $tRNA^{fMet}$, making it likely that EttA acts specifically on initiating ribosomes [62]. In vitro, EttA promotes formation of the first peptide bond of the elongation phase of translation [61, 62]. Endogenous EttA expression increases in stationary phase, and is required for normal competitiveness during extended incubation in stationary phase [61]. In the presence of high ADP/ATP ratios, EttA inhibits translation, most likely freezing the ribosome before the first peptide bond is made [61].

INTRODUCTION TO THIS THESIS

I am interested in the role that select proteins play in ribosome function. Towards this end, I performed a series of primarily genetic studies to unravel interactions between ribosomal protein L1, the GTPase BipA, and the ATPase Uup. In the chapters that follow, I will elucidate some of the effects on translation produced in the absence of L1 from the ribosome, and explore genetic interactions with the ΔL1 strain. I will also characterize a second member of the *E. coli* ABCF subfamily, Uup, and propose that Uup, like EttA, is an ATPase elongation factor in *E. coli.*

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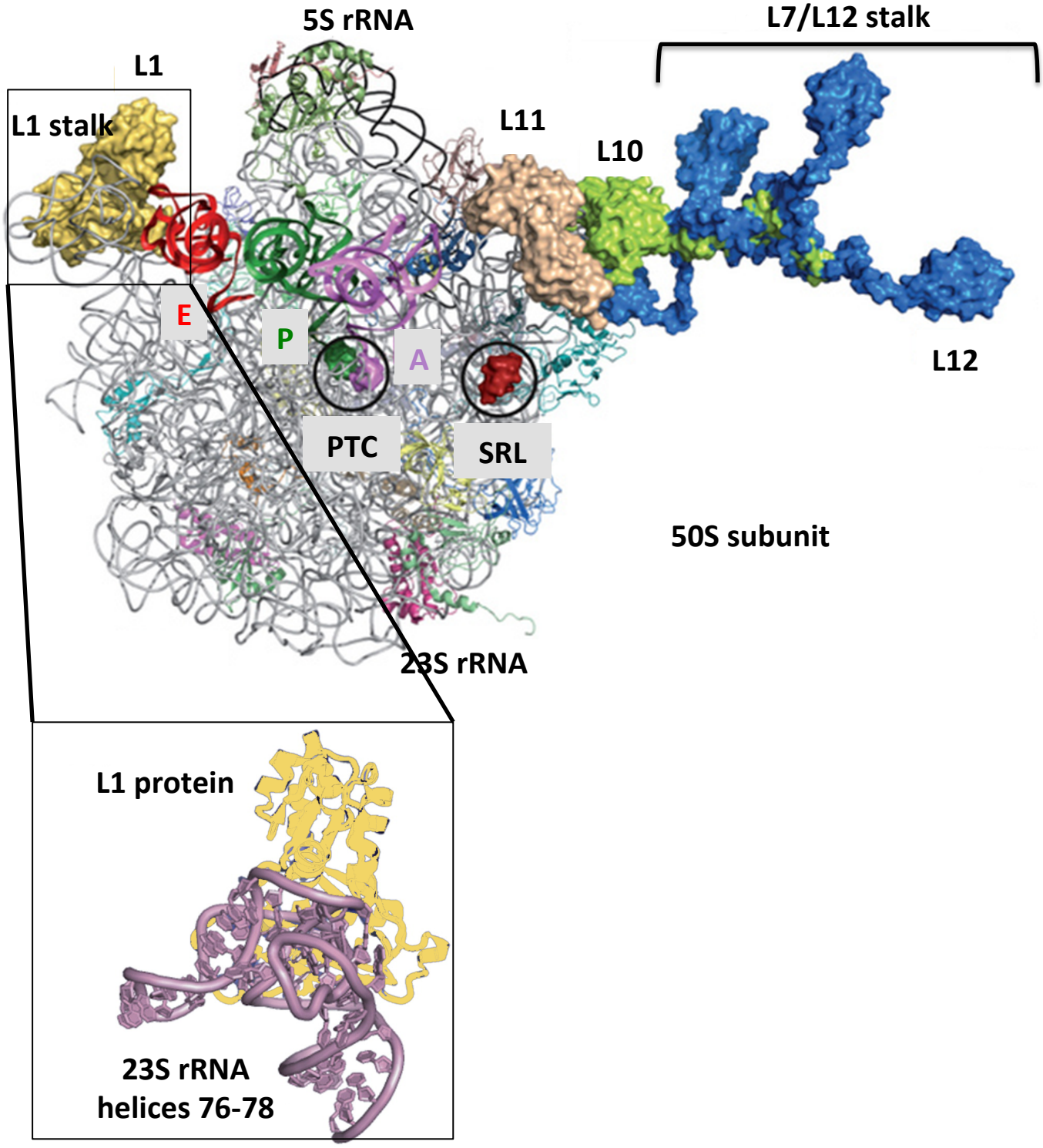

Fig. 1.1: Features of the large ribosomal subunit. The large ribosomal subunit is comprised of two rRNAs, the 23S (light grey) and 5S (dark grey) rRNAs The peptidyl transferase center (PTC, which catalyzes peptide bond formation) and sarcin-ricin loop (SRL, part of the binding site for translational GTPases) are labeled. The A, P, and E sites are shown occupied by tRNAs, and are also labeled. The two 50S stalks and their associated proteins are shown. Inset) Ribosomal protein L1 and its rRNA binding site. Modified from: Fei *et al.,* 2009; Yamamoto *et al.*, 2014

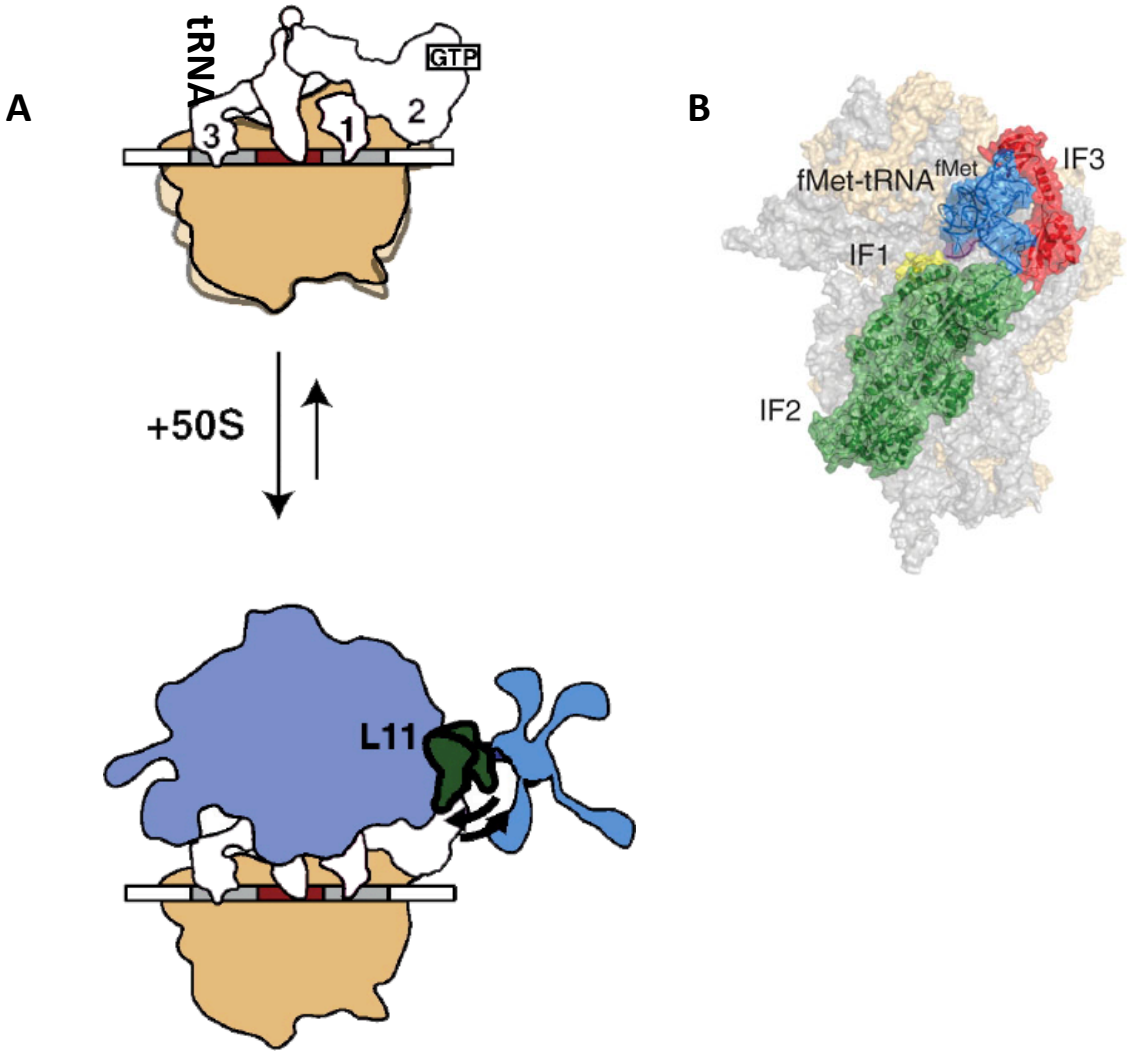

Fig. 1.2: Initiation of translation involves formation of a 30S pre-initiation complex and subsequent joining of the 50S subunit. A) Cartoon showing initiation factors and tRNA on 30S pre-initiation complex followed by joining of the 50S subunit. IF2 interacts with the large subunit in a region overlapping that of the elongation GTPases. IFs dissociate before elongation begins. B) Model of the 30S pre-initiation complex based on cryo-EM data.

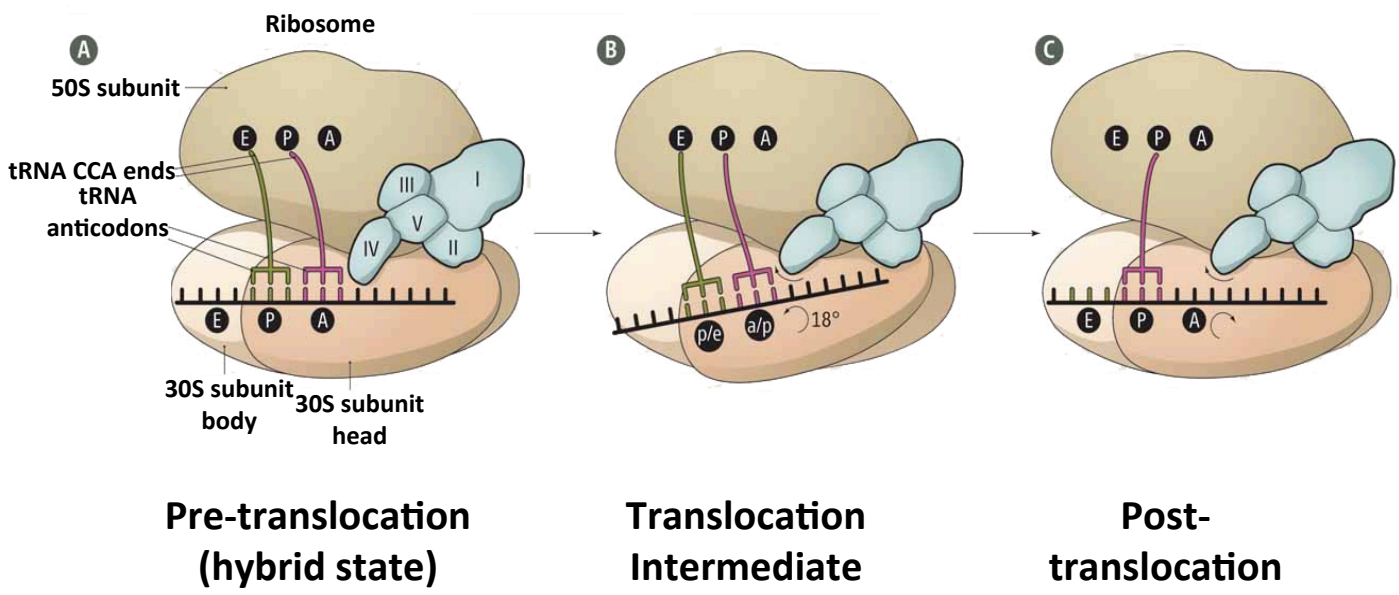

Fig. 1.3: EF-G catalyzed translocation. Translocation of deacylated and peptidyl tRNAs from the A and P sites to the P and E sites involves movement of both EF-G and the ribosome, particularly the 30S subunit. Many of the motions involved in translocation occur spontaneously and reversibly, but EF-G binding and GTP hydrolysis are required for the completion of translocation on a biologically relevant time scale. This figure shows how movement of the 30S body and head, along with domain rearrangement of Ef-G, contribute to translocation. A) A pre-translocation ribosome with tRNAs in hybrid states and EF-G*GTP bound. B) Rotation of the 30S subunit head facilitates translocation of the codon/anticodon interactions on the 30S subunit. C) Return motion of the 30S subunit head completes translocation. From: MacDougall and Gonzalez, 2015; Julian*et al.* 2011; Milon *et al.* 2012

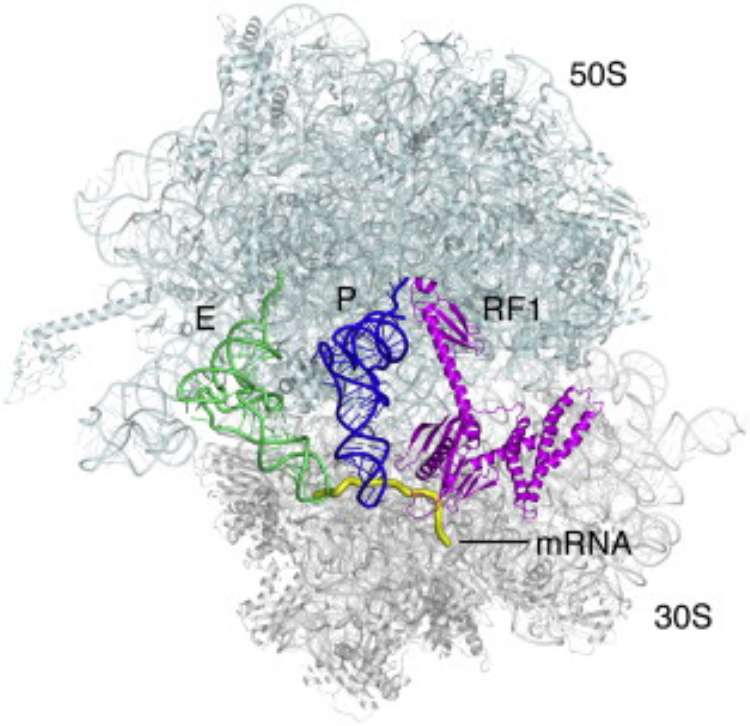

Fig. 1.4: Release Factors 1 and 2 interact with the stop codon in the A-site of the ribosome. The class 1 RFs, RF 1 and 2, bind in the A-site of the ribosome. Their unique tri-peptide motifs make contact with the stop codon and mediate their overlapping specificities. A conserved GGQ motif found in both class 1 RFs is located near the peptidyl transferase center and is involved in catalyzing peptide release. From: Loh and Song, 2010

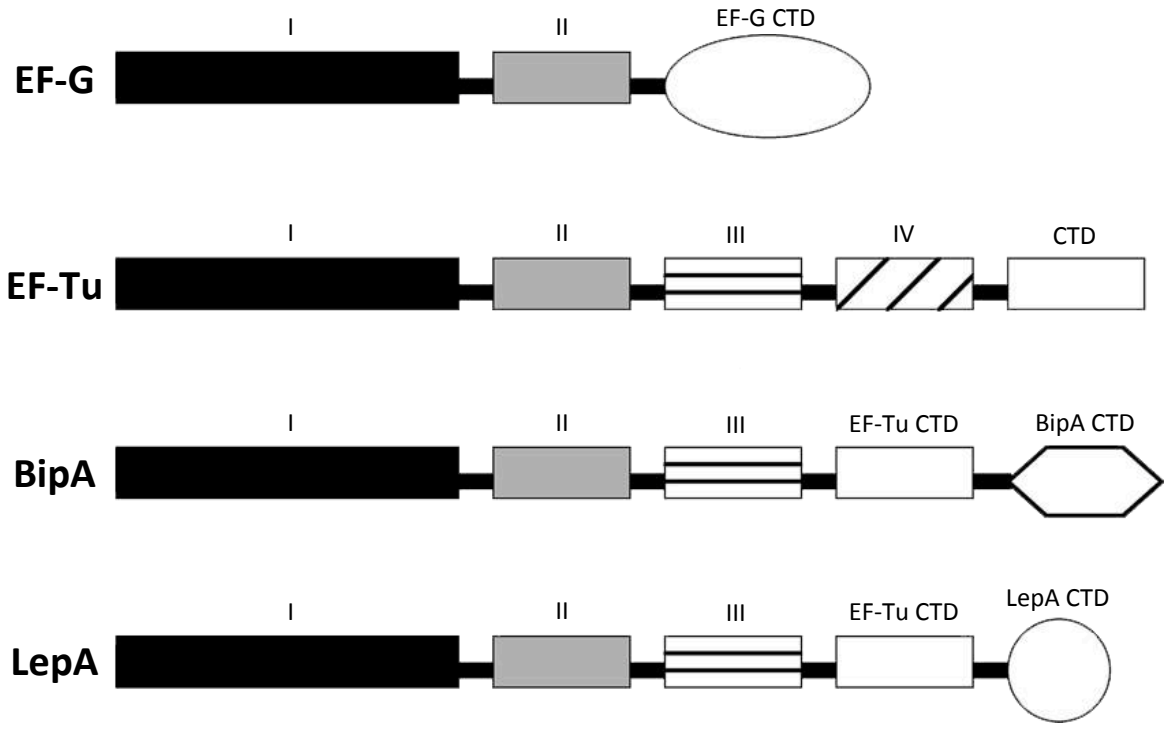

Fig. 1.5: Domain structure of elongation GTPase family EF-Tu, EF-G, BipA, and LepA comprise the elongation factor GTPase family. They share a related domain structure and bind at overlapping regions of the ribosome. EF-Tu binds aminoacylated tRNAs and delivers them to the ribosome. Domains III-CTD of EF-G, BipA, and LepA form structural mimics of the EF-Tu bound tRNA that have been shown or proposed to interact with the ribosomal A-site to mediate their distinct functions. Modified from Krishnan and Flower, 2008

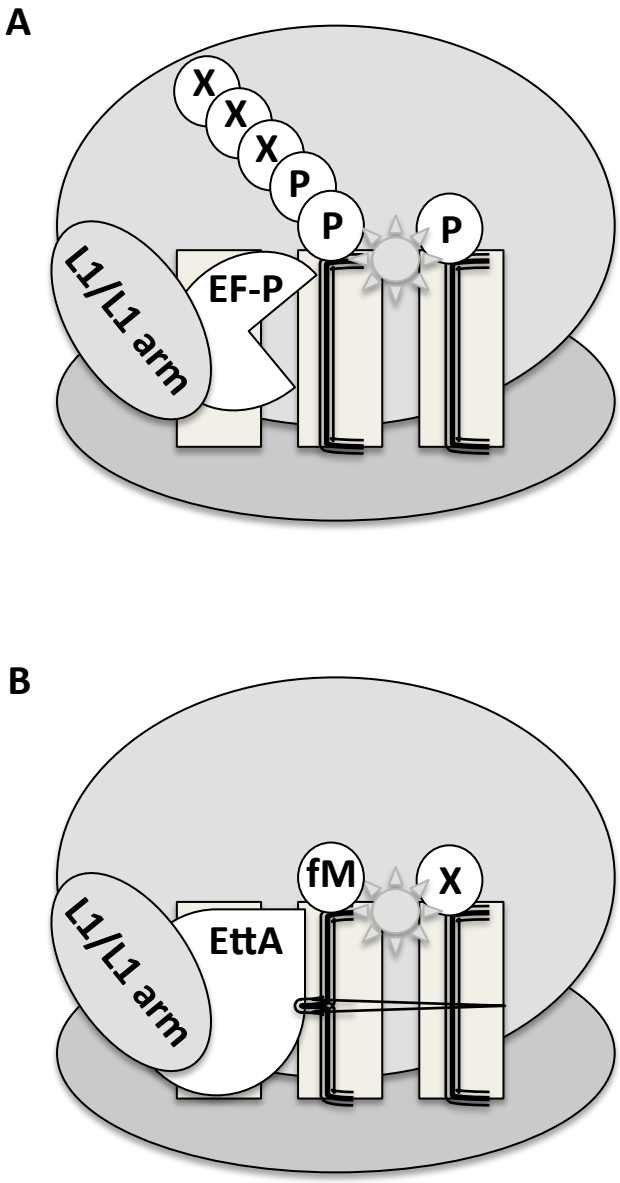

Fig. 1.6: E-site translation factors. A) EF-P and B) EttA are unrelated proteins known to affect translation by interacting with the ribosome in the E-site. A) EF-P promotes peptide bond formation when the ribosome encounters mRNA encoding certain stall-prone sequences, most of which involve successive prolines. B) EttA is an ABC-type ATPase that can promote or inhibit formation of the initial peptide bond, involving formyl-methionine on an initiator tRNA, depending on the ATP/ADP ratio in the cell.

Chapter 2

Uup is an ATPase involved in translation

ABSTRACT

Exogenous Uup suppresses many phenotypes caused by deletion of the elongation family GTPase BipA, including an OD_{600} dependent ribosome profile defect. Uup is an *E. coli* ABCF protein that shares many of the residues involved in binding to $tRNA^{fMet}$ and ribosomal protein L1 found in the closely related *E. coli* ABCF protein EttA. Both translation of reporter plasmids and translating ribosomes are reduced in Δ*uup*. While Δ*uup* and Δ*ettA* show different phenotypes in multiple assays, each can suppress defects resulting from loss of the other. Induction phenotypes of p*uup* and p*ettA* are similar in Δ*bipA* and Δ*rplA* (L1) backgrounds. We propose that Uup, as shown for EttA, can promote formation of the first peptide bond in the elongation phase of translation.

INTRODUCTION

The ABC (ATP-binding cassette) protein superfamily is large and consists of highly conserved proteins that carry out disparate functions. The signature feature of this

superfamily is the presence of two nucleotide binding domains that include a phosphate-binding loop (P-loop or Walker A motif) and a short consensus sequence "LSGGQ" [1]. The ABC modules can be encoded on a single protein or formed through dimerization. Based on the sequence similarity of the NBDs, the human ABC protein family was divided into seven subfamilies, denoted by the letters A–G [2]. The majority of ABC proteins are membrane bound primary transporters (for reviews, see [3-6]). This is not the case for the members of the ABCF family (also called the ART family; [7]). The ABCF proteins possess two ABC cassettes, lack transmembrane domains, and are involved in various aspects of translation (for reviews, see [4, 8, 9].

Saccharomyces cerevisiae encodes five ABCF proteins, eEF3, Gcn20, Arb1, New1 and Hef3. eEF3 is a fungal-specific essential elongation factor that interacts with eEF1A [10]. During elongation, the delivery of an aa-tRNA to the A-site is coupled with release of the deacylated tRNA from the E-site. eEF3 promotes the release of the E-site deacylated tRNA [11]. eEF3 has several N-terminal HEAT repeats, a four-helix bundle of unknown function, the two ATP-binding cassettes and a chromodomain insertion within the second ABC cassette [11]. In addition to its role in elongation, eEF3•ATP also catalyzes ribosome recycling [12].

Gcn20 is part of a part of a stress-responsive pathway activated by amino acid starvation (for reviews, see [13, 14]). The presence of uncharged tRNA in the ribosome decoding (A) site is detected by the kinase Gcn2 which phosphorylates

eIF2α (the α-subunit of eukaryotic translation initiation factor 2). Gcn2 binds to its effector protein Gcn1. Gcn20 is a protein required for full Gcn2 activation [15-17]. Gcn1, Gcn2, and Gcn20 may bind as a complex to the ribosome, allowing the deacylated tRNA in the A site to be delivered to Gcn2, resulting in Gcn2 activation and eIF2α phosphorylation[16, 18].

A model for the relationship between Gcn20 and eEF3 has been proposed [19]. During translation, eEF1A delivers the aa-tRNA to the A-site. This results in the eEF3-mediated loss of the deacylated tRNA from the E-site. The presence of eEF3 (specifically the HEAT domain and the C terminus) on the ribosome prevents the association of GCN1 and therefore, the entire Gcn1, Gcn2, Gcn20 complex from binding to the ribosome. In the presence of a deacylated tRNA in the A site, eEF3 binding is reduced, allowing for Gcn1, Gcn2, Gcn20 association and GCN2 kinase activation.

A mammalian ABCF protein related to Gcn20, called ABC50, is also involved in ribosome function [20]. The N-terminal domain of ABC50 that interacts with eIF2 and the ABC domains interact with the ribosome [21]. ABC50 is involved in start site selection as mutations in the ABC cassettes results in the increased usage of alternate start codons [22].

Less is known about the remaining 3 yeast ABCF proteins although they all are involved in ribosome function. Arb1, the yeast protein most similar to EttA, is an

essential protein required for biogenesis of both the large and small ribosomal subunits [23]. A *Δnew1* mutant is cold sensitive and has a 40S biogenesis defect [24]. Since the New1 protein is associated with translating ribosomes [24] the ribosome biogenesis defect could be indirect. New1 also binds to RNA in glucose-starved cells [25], perhaps indicating that it functions during stress. Hef3 is a non-essential paralog of eEF3 (84% identical) that arose from the whole genome duplication [26]. Hef3 is not expressed during vegetative growth but when expressed from a strong promoter, can substitute for eEF3 in vegetative cells [27]. Thus, Hef3 is functionally equivalent to eEF3 but functions under unknown growth conditions.

In *E. coli*, there are 79 ABC proteins, 4 of which are ABCF proteins[28]. These ABCF proteins, EttA (YjjK), Uup, YbiT and YheS, have all been predicted to also be involved in ribosome function [29]. Unlike many of the eukaryotic ABCF proteins, however, none of the genes encoding these proteins are essential in *E. coli*. Moreover, single deletions in each of these genes were reported to not have a striking phenotype in growth and survival at different temperatures, ability to grow on different carbon sources, sensitivity to antibiotics, sensitivity to UM radiation or motility [30].

EttA is predicted to be highly expressed in many bacteria [31, 32] and is the only *E. coli* ABCF protein for which a function is known. EttA has recently been shown by cryo-EM to bind in the E-site of the ribosome and contact both the L1 stalk

and the P-site tRNA [33]. EttA interacts with the distinctive features of the initiator $tRNA^{fMet}$, making it likely that EttA acts specifically on initiating ribosomes [33]. In vitro, EttA promotes formation of the first peptide bond of the elongation phase of translation [33, 34]. EttA is up-regulated in stationary phase, and is required for normal competitiveness during extended incubation in stationary phase [34]. In the presence of high ADP/ATP ratios, EttA inhibits translation, most likely freezing the ribosome before the first peptide bond is made [34].

In this chapter, I describe the characterization of another *E. coli* ABCF ATPase, Uup. Uup is the *E. coli* ABCF protein with the most sequence similarity to EttA. Uup was initially thought to be specifically involved in transposon movement as a *uup* mutant increases the precise excision for transposon Tn10 [35]. Mutations in *ssb*, *topA*, and *polA* also cause a similar precise excision phenotype for both Tn10 and mini Tn10 [35, 36]. Uup has a low intrinsic rate of ATP hydrolysis and a non-specific DNA-binding ability that is moderately reduced by deletion of its C-terminal tail [36, 37]. *uup* deletion mutants have a stationary-phase, cell-contact dependent viability defect when co-cultured with wild type cells [30]. No other growth defects of *uup* mutants have been reported. We identified Uup as a high copy suppressor of the cold sensitivity of the translational factor *bipA*. We therefore predicted that Uup would be a translational ATPase. Uup shares multiple amino acids with EttA that are important for binding to initiator-tRNA specific features of the tRNA and residues that interact with the L1 arm of the ribosome [34], making it quite possible that Uup also promotes peptide bond formation. Here, we will show evidence

supporting a role in translation for Uup, similar to that of EttA and ABCF proteins in other organisms.

METHODS

Bacterial strains, culture conditions, and growth measurements

E. coli strains used are listed in Table 2.1. Cultures were grown at the indicated temperatures in Luria-Bertani (LB) broth (10 g tryptone, 5 g yeast extract, 10 g NaCl per liter) or on LB agar plates (LB broth plus 1.5% agar) with antibiotics as appropriate. Culture growth was monitored by measuring the absorption at 600 nm. Antibiotics were used at the following concentrations: 100 μg/ml ampicillin (Amp), 20 μg/ml chloramphenicol (Chlor), 30 μg/ml kanamycin (Kan), 12.5 μg/ml tetracycline (Tet).

A mutantion, p*uupΔlinker*, that removes amino acids 294-300, including most of the second predicted RNA-binding region in the linker region of Uup, was created by cutting pCA24N*uup* with Nru1 and re-ligating. Other pCA24N-based *uup* mutant plasmids (D181N (*uup1*), D465N (*uup2*), D181ND465N (*uup*1,2)) were created using the original pBAD18 plasmids (kind gift of Dassa lab [30]). Mutant *uup* was amplified from pBAD18-*uup** (mutant) by PCR using forward primer (5'GCCTCATTAATCAGTATGCATGGC3') and reverse primer (5'CCGCCACCATTTTTTAACGC3'). A region containing the mutations of interest was excised using *Bgl*II and *Nsi*I and inserted into pCA24N-*uup* cut with the same enzymes. Transformed plasmids were sequenced to verify mutation.

Plate growth assay

Overnight cultures were serially diluted (1:10 dilutions) in 1 ml sterile water or LB media 7 times. The first dilution was discarded, and 5 µl samples of dilutions 2-6 were spotted onto LB-agar plates with the appropriate antibiotics in the presence or absence of 25µM IPTG or .02% arabinose, as appropriate. Plates were incubated at the temperature indicated until the complemented strains reached an equivalent size. Pairs of plates containing the same dilutions with and without IPTG were incubated and removed together.

Western Blot Analysis

Proteins were separated by SDS-PAGE and transferred to nitrocellulose in a Hoefer TE77 semidry transfer apparatus. Membranes were blocked and probed in 5% skim milk in phosphate buffered saline (137mM NaCl, 12 mM phosphate, 2.7 mM KCl, pH 7.4; PBS) with 0.1% TWEEN20 (PBST) using a 1:1000 concentration of affinity-purified polyclonal rabbit anti-BipA or a 1:500 concentration of affinity-purified polyclonal rabbit anti-Uup. Blots were rinsed in PBST (anti-BipA) or PBS (anti-Uup). Bound antibody was detected using a 1:20,000 dilution of horseradish peroxidase-conjugated goat anti-rabbit and visualized on IBF Blue X-Ray film using house-made ECL [38]. Protein levels were quantified from western blot using ImageJ.

Ribosome Profiles

Cultures were grown at 37°C or 18°C until they reached an OD_{600} of 0.350-0.800. Cells were pelleted and prepared for sucrose density gradient centrifugation as previously described [39] except that $MgAc_2$ was used instead of $MgCl_2$ and chloramphenicol was omitted where stated. Approximately 13 OD_{260} units were loaded on top of either 4.95 ml 7%-47% sucrose gradients (Fig. 2.5, 2.6, 2.10) or 10 ml 23% sucrose freeze-thaw gradients (Fig. 2.12). 4.95 ml gradients were centrifuged at 41000 rpm for 1.5 hours in a Beckman SW50.1 rotor. 10 mL gradients were centrifuged at 35,000 rpm for 2.5 hours in a Beckman SW41 rotor. After centrifugation, gradients were fractionated as in [40] using a flow rate of .350 ml/min for the 4.95 ml gradients or .750 ml/min for the 10 ml gradients. Profile data was captured digitally using a computer connected to the ISCO UA-5 detector.

Antibiotic Sensitivity Assay

Cultures were grown overnight under selection, then 100 µl cultures diluted 1:2 were spread-plated on LB agar plates with the appropriate antibiotics and 25 µM IPTG. Antibiotic disks (Rif 5 µg) were placed on plates and plates were incubated at 18°C for 4 or 5 days, until a lawn was established.

β-galactosidase Translation Assay

Overnight cultures of wild type, Δ*uup*, and Δ*bipA* strains harboring pSG*lacZ*–based reporter plasmids [41]were grown under selection then back-diluted 1:50 into 4 ml LB with appropriate antibiotics. Cultures were grown at 18°C or 37°C until they reached OD_{600} 0.25-0.65. Production of β-galactosidase was assayed as in [41].

Protein and Ribosome Purification

For protein purification, overnight cultures of JM 6052 were diluted 1:100 in 1.5 L LB and grown at 37°C for 4 h, and then induced with 200 μM IPTG for 2 h. Cells were pelleted and resuspended in lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole, pH8) and lysed by 4 passages through a French pressure cell at 1,200 psi. Lysates were centrifuged for 30 min at 10,000xG to clarify. The cleared lysate was incubated with 4-ml Ni-nitrilotriacetic acid (NTA) (Qiagen) bead slurry with gentle shaking for 45 min at 4°C. The column was allowed to settle, then was washed with 5 bed volumes of lysis buffer followed by 5 bed volumes of wash buffer (lysis buffer with 20mM imidazole). The protein was eluted in 10 ml of elution buffer (lysis buffer with 100mM imidazole). Aliquots from fractions were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on a 10% SDS-polyacrylamide gel, and proteins were detected by Coomassie blue stain. Fractions containing Uup were pooled, filtered (0.22 μm) to clarify, and dialyzed against storage buffer (10% glycerol, 20 mM Tris pH 6.8, 100 mM NaCl, 1 mM β-mercaptoethanol) in an Amicon YM-50 centriprep filter (spin 5 min at 1000xG, dilute filtrate with storage buffer to 4 ml volume-repeat 3 times). Pooled fractions were further purified by FPLC using toyopearl DEAE-G50 resin with 50 mM to 1 M NaCl. Protein was detected by separating aliquots by SDS-PAGE as before and fractions containing significant protein were pooled and frozen.

Ribosomes were isolated from BW25113. Cultures were grown to midlog phase, then pelleted by centrifugation at 6,500 rpm for 10 min at 4°C in a F145-6x500y rotor (Sorvall). Pellets were resuspended in NB buffer (10 mM Tris-Cl pH 7.7, 60 mM NH_4Cl, 1 mM $MgAc_2$) then lysed by sonication (10 sec bursts followed by 10 sec rest on ice for 5 cycles) with a Kontes Micro Ultrasonic Cell Disruptor. Lysate was cleared by centrifugation at 10,000xG for 30 min at 4°C. Supernatants were applied to a 1.1M sucrose cushion (20 mM Tris-Cl, pH7.5, 10 mM $MgCl_2$, 50 mM NH_4Cl) and subjected to ultracentrifugation in an SW50.2 rotor (Beckman) at 23,000 rpm for 18 h at 4°C. Pellets were resuspended in NB buffer and frozen immediately.

Ribosome binding assay

Purified Uup (25 pmol) was mixed in NB buffer with or without 42.6 pmol purified ribosomes and either 20 mM ATP, ADP, AMP-PNP, or GTP, or no nucleotide. Reaction mixtures were incubated on ice for 30 min then centrifuged at 383,000xg for 40 min in a TLA-100 rotor (Beckman) to pellet ribosomes. The supernatant was removed, and pellet was resuspended in 100µL of water. The supernatant and pellet fractions were analyzed for the presence of Uup by western blot. Western blots were quantified using ImageJ.

Stationary phase viability assay

Overnight cultures were diluted 1:100 in LB +chlor and grown to either mid log or stationary phase in the presence of 25 µM IPTG. Equivalent OD_{600} units were plated

onto media also containing 25 μM IPTG and the cfu/ml were calculated for each strain.

RESULTS

Mutations in genes encoding non-essential proteins involved in translation often result in cold sensitive slow growth [42, 43]. This is true for the poorly characterized *bipA* gene[44]. Although BipA shares sequence similarity to the GTPases EF-G, EF-Tu and LepA, the function of BipA is unclear. We carried out a screen to identify high-copy suppressors of the Δ*bipA* cold-sensitivity. The most robust suppressor was Uup. Induction of p*uup* with 25 μM IPTG in the Δ*bipA* background at 18° results in colonies that are nearly wild type in size (Fig. 2.1A). Thus, the ATPase Uup is a bypass suppressor of the GTPase BipA.

In order to determine whether the expression pattern of these proteins is temperature-dependent, we assayed the amount of endogenous BipA and Uup found in exponentially growing cells at 37 °C and 18 °C (Fig. 2.1B). We found that BipA was expressed approximately 2-fold more strongly in the cold. However, Uup expression did not change in a temperature-dependent manner. It was previously noted that Uup levels do not change with different phases of growth [36]. Since Uup induction from a non-native promoter resulted in suppression of the Δ*bipA* phenotype, we tested whether BipA is required for Uup expression. In a *ΔbipA* strain, Uup is expressed at higher levels than in a WT strain at both 37 °C and 18 °C (Fig 2.2B) (approximately 2 and 10-fold, respectively). Thus, while Uup expression

is not temperature-dependent in wild type cells, endogenous Uup levels are elevated in a Δ*bipA* mutant and the extent of elevation is temperature dependent. This is interesting in light of the role of Uup as a high copy suppressor of Δ*bipA* cold-sensitive mutant phenotype, as it indicates that the cell is already producing higher levels of Uup in response to the lack of BipA.

The Uup protein has 4 distinct domains: ABC1, the linker region, ABC2, and the C-terminal domain (CTD). In order to determine the importance of each of these regions for Uup function, we examined strains carrying IPTG-inducible plasmids expressing *uup* mutants, including ATPase-null mutations in one [p*uup1*, (D181N); p*uup2*, (D465N)] or both [*puup1,2*, (D181N/D465N)] ABC cassettes and mutants lacking the linker region [p*uupΔlinker*, (Δaa294-300)] or CTD [p*uupΔCTD*, (V550STOP)]. The ABC cassette mutations have been previously characterized and result in the lack of ATPase activity in the respective Uup ABC cassette [36].

First, we asked whether there was any consequences on cell growth at 37° or 18° when these plasmids were introduced into wild type cells (Fig 2.2). In all case, cell growth a 37°C of strains with *uup* plasmid variants was similar to that of wild type cells harboring an empty vector, regardless of the addition of IPTG. We did note a slight growth sensitivity of cells expressing the p*uup2* plasmid. At 18° however, the presence of p*uup2*, but not the other p*uup* variants, resulted in a significant reduction in growth. This dominant negative effect occurred with or without induction.

We next asked whether expression of the plasmid-born *uup* variants as the sole copy of *uup* in the cell would result in a growth defect (Fig. 2.3, lower panel). We tested the effect of over-expression of the *uup* variants in a *Δuup* background. In contrast to what was seen in the wild type background, overexpression of Uup2 and Uup1,2 were detrimental at 37°C in the Δ*uup* background. In the cold, overexpression of both Uup2 and Uup1,2 resulted in striking slow growth. Moreover, expression of the Uup-Δlinker variant also resulted in slow growth in the cold.

Given that overexpression of wild type Uup suppresses the Δ*bipA* cold-sensitive growth phenotype, we wanted to determine (1) whether both ABC cassettes, the linker or C-terminal domains were important for this suppression and (2) whether the presence of BipA was important for the observed growth defects when select *uup* mutants were expressed (Fig 2.3). In a *ΔbipA* mutant background, expression of the mutant *uup* variants at 37° had little effect on cell growth. Overexpression of the Uup1 mutant suppressed *ΔbipA* as well as wild type Uup at 18° indicating that ATPase activity of the first ABC cassette is not critical for the observed suppression. *ΔbipA* mutants harboring p*uup2* were partially suppressed in the absence of IPTG and did not grow in the presence of IPTG demonstrating that the overexpression dominant negative phenotype of this *uup* mutant does not rely on BipA. The *uup*1,2 double mutant and the *uup*ΔCTD showed modest suppression of the Δ*bipA* slow growth in the cold in the presence, but not absence of IPTG. Finally, as seen in the

Δ*uup* mutant, but not the wild type strain, even uninduced expression of the linker domain mutant resulted in a severe growth defect. The presence of either BipA or Uup suppresses this synthetic lethality as no growth defect was seen when *uup*Δlinker was expressed in wild type cells (Fig 2.2).

To eliminate the contribution of wild type Uup in our studies, we also examined a Δ*uup*Δ*bipA* mutant harboring the *uup* variants (Fig 2.3, bottom panels). We found that this double mutant exhibited a cold-sensitive growth defect more severe than seen with either single mutant. This observation is consistent with the elevated Uup levels seen in Δ*bipA* at 18°C (Fig. 2.1). At 37°, none of the plasmids resulted in significant cell growth differences with the exception of p*uup2* which had a modest effect when overexpressed (Fig 2.3). As seen in the Δ*bipA* mutant containing a chromosomal copy of *uup*, Uup1 suppressed Δ*bipA* cold sensitivity in the presence of IPTG whereas Uup2 partially suppressed Δ*bipA* in the absence of IPTG but resulted in no growth in the presence of IPTG. Without endogenous Uup, strains carrying *uup1,2* , *uupΔCTD* or *uupΔlinker* variants grew as poorly as with empty vector.

Previously, BipA was shown to localize to either the 30S or 70S ribosome, depending on the growth conditions [45]. We compared the ribosome association pattern of BipA and Uup in wild type cells. We found that BipA associated across the ribosome fractions, including on polysomes, with higher densities at the 30S and 70S peaks and at the top of the gradient (not associated with ribosomal particles)

(Fig 2.4B). Uup had a similar pattern to that of BipA, with higher levels in 30S, 50S, and 70S fractions and low levels of association throughout the polysome fractions.

We thought that the dominant negativity of Uup variants shown in Fig. 2.2 and Fig 2.3 might have to do with their ribosome binding. In order to test this possibility, wild type strains carrying each mutant *uup* plasmid and EV were grown under inducing conditions and the cell extract subjected to sucrose gradient centrifugation. The Uup content of resulting fractions was analyzed by western blotting. We found that both variants that are dominant negative in a wild type background associated with the ribosome to a higher degree than the variants that are not dominant negative (Fig. 2.5). This suggests that the dominant negative phenotypes of Uup2 and UupΔlinker are due to continued, inappropriate occupancy of the ribosome. Sustained Uup occupancy of the ribosome would inhibit continued translation, by my model.

Dual ABC cassette proteins such as Uup change their conformation when they bind ATP [46]. For ribosome-associated ABC proteins, this conformational change often means that the strength of the ribosome association can depend on whether the protein is complexed with ATP or ADP [47]. For instance, it has been proposed that EttA promotes the first peptide bond when ATP levels are high, but binds tightly to the ribosome and suspends elongation in the presence of high ADP [34]. In order to determine whether Uup has a nucleotide preference when bound to the ribosome we assayed ribosome binding of Uup in the absence of nucleotide, and in the

presence of ATP, non-hydrolysable analog, ADP, or GTP (Fig 2.5A). In this pelleting assay, we found that Uup bound ribosomes without the addition of nucleotide. The presence of ATP, however, resulted in higher levels of ribosome binding (~2x). The lack of a similarly strong binding in the presence of non-hydrolysable ATP analog is puzzling. Uup may perform its ribosome-associated function when cellular energy is high and translation is actively occurring.

Recently, it was published that a Δ*bipA* mutant has a polysome defect at 18°C [48], an observation we had also made in our lab (unpublished data). At low temperature, *ΔbipA* mutants have reduced mature 50S particles, accumulate a 32S peak and have reduced polysomes (Fig 2.6). As BipA is most like the translational GTPases, it is likely that this defect is due to the misexpression of a key ribosomal protein or ribosome assembly factor. In wild type cells, the ribosome profile is similar at early, mid, and late log growth phases (top panel). In Δ*bipA* cells, the ribosome profile defect is cell density dependent and begins to lessen as the culture approaches late logarithmic growth phase (Fig 2.6). Compared to Δ*bipA* mutants harboring an empty vector, the polysome profiles of *ΔbipA* mutants carrying p*uup* were less perturbed and more similar to wild type profiles at earlier optical densities. Thus, Uup partially suppresses the Δ*bipA* ribosome profile defect.

Although BipA is a translational GTPase both by family and by ribosome localization [49, 50], Δ*bipA* mutants are also sensitive to the transcriptional inhibitor rifampicin at 18°C (Fig. 2.7). Induction of plasmid-borne Uup suppressed the sensitivity

completely (Fig. 2.7). Because it is not known whether BipA is functionally similar to either of the related translational GTPases EF-G (encoded by *fusA*) or LepA, we tested whether over-expressing either of these GTPases could suppress the Δ*bipA* rifampicin sensitivity. Over-expression of EF-G partially reduces the Δ*bipA* rifampicin sensitivity whereas LepA does not.

BipA is most similar to translational GTPases. To determine whether BipA plays a role in translational fidelity we examined the fidelity of translation from a set of LacZ reporter constructs designed to determine (1) the initiation codon used, (2) the fidelity of an appropriate stop codon and (3) the ability to frameshift [51]. In wild type cells, AUG is preferentially used over the two other *bona fide* start codons, UUG and GUG at either 37° or 18°. We observed the same preference for the AUG codon in the *ΔbipA* mutant (Fig 2.8A). Thus, BipA is not likely required for normal start site selection (P-site selection) during translation initiation. This was also the case for utilization of non-canonical start codons (Fig 2.8B). At either temperature, both wild type and Δ*bipA* mutants show low-level utilization of the non-start codons. To determine whether BipA plays a role in stop codon selection (A-site selection), we examined a series of strains with a premature stop codon. Both wild type and *ΔbipA* mutants showed a low read through of the stop codons. There was a slight decrease for UGA read-through in the *ΔbipA* mutant, primarily at 37°C (Fig 2.9D). We also note an increase in fidelity through both -1 and +1 frameshift mutations at 37°C, but not at 18°C. Taken together, we did not ontice any striking consequences on the fidelity of cells lacking BipA.

We also tested the Δ*uup* strain for defects in bulk translation, P-site specificity, and A-site specificity. The Δ*uup* strain showed a general decrease in translation from all three alternate start codons at 37° (Fig. 2.9A). This defect is complemented by addition of a plasmid expressing *uup*. Translation efficiency is also restored by introduction of plasmids encoding low levels of the Uup1, Uup2 or ΔCTD mutants. The Uup1,2 double mutant, however, does not complement (Fig 2.10A). Thus, one or the other ABC cassette is important for translation initiation efficiency. Curiously, at 18°C, we see no difference between wild type and Δ*uup* in translation from canonical start codons (Fig. 2.10A).

In general, there was little difference in initiation from non-start codons between Δ*uup* and wild type strains at 37°C (Fig. 2.9B), although the mutant had increased initiation from the AUA codon and slightly decreased translation from an AUU codon. There is a slightly more pronounced effect seen at 18°C, with increased initiation seen in the mutant at both the CUG and AUA codons. At both temperatures, strains containing p*uup* also showed some altered initiation, which may be an over-expression phenotype. The Δ*uup* strain showed some change in A-site fidelity as measured by stop codon read-through and frame-shifting at both temperatures (Fig. 2.9C), but the effects of over-expression were more striking, particularly at 37°C. At 18°C, the most prominent changes were the about halving of UGA read-through and of +1 frameshifting. However, neither of these changes were complimented by p*uup*. The lack of complementation may be due to an over-

expression phenotype. Overall, we do not see dramatic effects on translation fidelity in the Δ*uup* strain.

In light of the interaction of Uup with the ribosome, and the ability of Uup to suppress the Δ*bipA* ribosome profile defect, we tested whether a Δ*uup* mutant shows a ribosome assembly defect (Fig. 2.10, top panels) at either 37°C or 18°C. We found no evidence for a ribosome assembly defect in *Δuup* cells. We found, however, a slight decrease in polysomes in the Δ*uup* deletion strain, which was complementable by addition of p*uup*.

To more clearly resolve differences in levels of translating ribosomes, samples were prepared without the addition of chloramphenicol, which blocks the exit channel of the ribosome and prevents continued translation, allowing translating ribosomes to run off the mRNA. Wild type ribosome profiles prepared in the absence of chloramphenicol show the expected small reduction in polysomes. In the absence of Uup, however, there was a striking decrease in mRNAs loaded with multiple translating ribosomes (2.10, bottom panels). This supports the findings reported in figure 2.8A, showing a decrease in translation in the absence of Uup.

Uup is one of a four dual ABC cassette proteins in the *E. coli* ABCF family [28]. Figure 2.11A depicts the relationships of *E. coli* and *S. cerevisiae* ABCF proteins and RbbA, a non-ABCF *E. coli* ABC protein [52-54]. Uup is most similar to the protein EttA, which

has recently been characterized [33, 34]. We asked whether these other *E. coli* ABCF proteins could suppress the Δ*bipA* slow growth in the cold (Fig. 2.11B). While Uup is the strongest suppressor of the Δ*bipA* cold sensitivity, high copy EttA was also able to suppress to a lesser degree. Neither high-copy Ybit nor high-copy YheS suppressed the Δ*bipA* cold sensitivity. In fact, Δ*bipA* grew more slowly in the presence of p*ybiT* or p*yheS* compared to in the presence of empty vector. In wild type cells, presence of p*ybiT*, but not the other plasmids, resulted in slightly reduced growth at 18°C. This suggests that high-copy Uup and high-copy EttA can produce overlapping effects *in vivo.*

EttA makes contact with the L1 stalk when bound to the ribosome [33]. Cells lacking *rplA* (encoding L1) grow slowly. We assessed the consequences of over expressing *E. coli* ABCF proteins in a Δ*rplA* mutant (Fig. 2.11B). We found that high-copy Uup, and, to a lesser extent, EttA and BipA resulted in still slower growth in the Δ*rplA* background. The similar effects of high-copy EttA and Uup (and BipA) are sensitive to the presence of L1 in the cell.

The similar phenotypes seen upon induction of *uup* and *ettA* in in Δ*bipA* and Δ*rplA* in figure 2.11B led us to further explore potential similarities between Uup and EttA (Fig 2.12). First, we tested whether a Δ*ettA* strain shows a decrease in translating ribosomes when assayed in the absence of chloramphenicol, as seen for the Δ*uup* strain. The Δ*ettA* ribosome profile does not show a striking reduction in polysomes (Fig. 2.12A, top panels), however, there is a decrease in the 70S peak with a

concomitant increase in both 30S and 50S peaks, suggesting dissociation of the 70S ribosome into its component subunits. p*uup* suppresses the Δ*ettA* profile abnormality (Fig. 2.12A, middle panels). Similarly, expression of *ettA* is able to suppress the Δ*uup* polysome reduction (Fig. 2.12A, bottom panels). Thus, high copy Uup and EttA are able to at least partially substitute for each other functionally. We do not believe that they are functionally redundant, however, as their ribosome profile defects are clearly different. Deletion of *uup*, but not *ettA*, results in decreased translation from AUG of the *lacZ* reporter plasmid (Fig. 2.8A, Fig. 2.12B), further supporting unique endogenous roles for Uup and EttA.

EttA is expressed primarily in stationary phase, and loss of EttA impairs cell survival in stationary phase [34]. Uup expression does not vary with growth phase [36]. We tested the effect of both loss and over-expression of Uup on cell viability. We found that Uup is not required for viability in late or mid exponential phase at either 37 or 18°C (Fig. 2.13). Wild type cultures expressing p*uup*, however, showed decreased cell viability of aproximately 2-fold and 10-fold for mid and late log cells (Fig. 2.13A). This effect was temperature independent. Thus over-expression of Uup is most deleterious under the conditions where EttA is required for normal survival.

Uup was originally reported to be a DNA-binding protein involved in transposon excision because its deletion results in increased precise excision of transposons, a phenotype seen in mutations of genes encoding DNA-binding proteins such as *ssb* and *topA* [35, 55, 56]. The evidence we present in this document, however, shows

clearly that Uup associates with the ribosome. If Uup promotes translation in the cell, then the Δ*uup* transposon excision phenotype could be due to reduced translation of one or more of these DNA-binding proteins. If this is the case, then increasing the level of Uup in a cell might be able to suppress partially functional mutations in one or more of these genes. Therefore, we tested the ability of plasmid-encoded Uup to suppress the temperature sensitivity of a *topA* mutation.

The Δ75*topA* strain containing pBAD*uup* grew at the restrictive temperature (Fig. 2.14A). Growth of the mutant containing pBAD*uup* appears slightly better in the presence of inducer than in its absence. The Δ75*topA* strain is prone to spontaneous suppression, which has caused difficulty in independently verifying this finding. In order to better understand the interaction of Uup and TopA, we assayed the levels of Uup in wild type and Δ75*topA* strains. There appears to be increased levels of Uup in the mutant strain than in wild type cells (Fig. 2.15C), suggesting that the tentative suppression of Δ75*topA* may be accurate and relevant.

DISCUSSION

The role of the EF-G family GTPase BipA, like that of its family member LepA, has been difficult to determine. BipA contains the ribosome binding domains seen in its family members LepA, EF-G and EF-Tu [57] and competes with EF-G for ribosome binding, suggesting that it will also bind on the conserved binding region of this family on the A-site side of the ribosome [58]. Other experiments indicate that BipA protects the A-site mRNA [59], suggesting that a portion of BipA (likely its unique C-

terminal domain) can occupy the ribosomal A-site, as expected if BipA functions as a translation factor. A crystal structure published during editing of this document shows that BipA does indeed interact with the ribosome on the A-site side, and can bind to ribosomes containing tRNA in the A, P, and E sites [50]. The ribosome assembly defect seen in Δ*bipA* strains when grown in the cold raised the question of whether BipA could also directly affect ribosome assembly [48]. An alternative possibility is that one or more ribosome assembly factors are misexpressed in Δ*bipA* mutants.

The OD_{600} dependence of the Δ*bipA* 50S ribosome assembly defect seen in cells grown in the cold is strikingly similar to the OD_{600} dependent 30S ribosome assembly defect seen in Δ*lepA* strains grown in acidic conditions, where LepA is needed for normal cell growth [60]. This, combined with our unpublished results implicating BipA in the efficient translation of certain mRNAs, provides strong support that the Δ*bipA* ribosome profile defect seen in the cold is secondary to a translation defect of one or more messages important for normal ribosome assembly. Further, the temperature-dependence of the defect, combined with BipA temperature-dependent expression, suggests that a cold temperature is one condition where endogenous BipA is likely to function. This would mean that while two of the elongation factor GTPases (EFG and EF-Tu) are important for translation in all conditions, the other two members of this family (LepA and BipA) are reserved for stress conditions.

The synthetic effect on growth seen in the Δ*bipA*Δ*uup* double mutant suggests that Uup and BipA promote similar processes within the cell by different means and/or pathways. Uup has been previously shown to bind DNA non-specifically [37]. However, both sequence-based predictions and functional characterizations of other ABCF proteins [20, 33, 34, 47] suggest RNA is more likely to be the functional binding partner of Uup, a conclusion corroborated by our finding of Uup on translating. The decrease in translating ribosomes seen in the absence of Uup together with the decrease in translation from the AUG-start reporter plasmid further support the hypothesis that Uup promotes translation, as hypothesized for BipA.

Uup is most similar to its family member, EttA. Our results show that over-expressed EttA behaves similarly to over-expressed Uup in two different genetic backgrounds, although Uup has a stronger effect in both backgrounds (Δ*bipA* and Δ*rplA)*. Also, induction of plasmids encoding EttA or Uup in Δ*uup* or Δ*ettA* mutants results in cross-suppression of the ribosome profile defect of the other mutant. Δ*uup* and Δ*ettA* strains, however, have very different ribosome profile defects, and the Δ*bipA*Δ*ettA* strain does not show the synthetic sickness of the Δ*bipA*Δ*uup* strain (data not shown), indicating the proteins do not independently affect the same process. Also, while EttA is important for long-term survival in stationary phase, the Δ*uup* strain shows no viability defect in late exponential phase. Indeed, induction of plasmid-borne Uup reduces cell viability that is most pronounced as cells enter stationary phase. Finally, unlike the Δ*uup* strain, deletion of *ettA* produced no

detectable decrease in translation of the AUG-start reporter plasmid. Taken together, it appears that Uup and EttA can functionally substitute for each other at high copy, but normally they play slightly different roles in the cell.

A critical look at the properties of EttA may help us to resolve these seemingly contradictory facts. EttA binds in the E-site of the ribosome and contacts L1, the L1 arm, and the initiator $tRNA^{fMet}$ in the P-site [33]. *In vitro,* EttA promotes the formation of the first peptide bond and then leaves the ribosome under conditions where the ADP:ATP ratio is low. However, when the ADP:ATP ratio is high, EttA binds strongly and persistently to the ribosome without promoting peptide formation, inhibiting elongation and sequestering a set of ribosomes poised to rapidly re-enter the elongation phase [33, 34]. The endogenous EttA expression is low during exponential phase, and increases significantly during the transition to stationary phase, when cellular energy levels decrease [34]. Thus, the primary role for EttA should be to pause translation and sequester ribosomes. This leaves the role of promoting formation of the first peptide bond when ATP:ADP ratios are high without an occupant. Perhaps Uup is the primary ATPase performing this role in exponentially growing cells.

Uup contains four of the six residues in EttA predicted to interact with $tRNA^{fMet}$ in the P-site, and also has significant homology with EttA in the L1-interacting "arm" region [33] (Fig 2.15A). The functional relevance of these sequence similarities is borne out by the ability of Uup and EttA to suppress each other's ribosome profile

deletion defect and further supported by the similar phenotypes upon induction of p*uup* and p*ettA* in the Δ*bipA* and Δ*rplA* backgrounds. In keeping with the demonstrated ability of EttA to promote translation under conditions when it not naturally highly expressed [34], exogenous production of EttA during exponential phase can restore levels of translating ribosomes decreased in the absence of Uup. The ability of plasmid-borne Uup to suppress the Δ*ettA* ribosome profile phenotype suggests that Uup can also pause translation at high copy. To us, these data suggest that the primary role of Uup in the cell is to promote the first peptide bond of elongation in exponential phase cells, most likely in a select subset of mRNAs or during a specific physiological state (Fig 2.15B). As EttA levels increase during the transition to stationary phase, EttA bound to ADP would out-compete Uup for these ribosomes and sequester both the ribosomes and the mRNA around which they are assembled until conditions are once again favorable for rapid growth. A role for Uup in promoting translation would explain Uup suppression of the deletion phenotypes of the elongation factor BipA as well as the decrease in translation seen in the Δ*uup* mutant.

The data showing the effect of loss and excess of Uup on cell viability is easily understood in the context of this hypothesis. Loss of Uup results in no apparent effect on cell viability at either temperature or growth phase, perhaps because the subset of mRNAs Uup affects is not important for viability under these conditions or perhaps because the action of other elongation factors, such as BipA, are able to compensate for the lack of Uup. Induction of Uup results in a mild effect on viability

during exponential phase, perhaps because of excess translation of some message not usually translated in this phase, or due to interference with BipA function. The significant viability defect seen upon induction of p*uup* as cells enter stationary phase, however, could be attributed to competition of Uup with EttA for ribosome binding and resulting interference with EttA-mediated remodeling of translation that usually helps cells to cope with the scarcity encountered during stationary phase.

A role for Uup in promoting translation by encouraging formation of the first peptide bond raises the tantalizing possibility that the previously-reported transposon excision (*tex*) phenotypes of Δ*uup* strains are due to inactivation of translation of one or more of the other reported *tex* genes in the absence of *uup*. Our results show that induction of Uup does not suppress the temperature sensitivity of a mutation in one tex gene, *ssb*. However, plasmid-borne Uup may suppress the temperature-sensitive growth phenotype of mutation of another *tex* gene, *topA*. This possibility must be viewed with caution due to the interference of spontaneous second site suppressors in verification attempts.

In summary, we propose that Uup is an E-site factor important for formation of the first peptide bond of the elongation phase of protein synthesis for a subset of mRNAs or under certain physiological conditions(Fig. 2.15B). We also expect the elongation factor GTPase BipA to promote translation in certain cases, by action at

the A-site of the ribosome. Our data supports the hypothesis that the effect of these two proteins on translation can be synergistic.

Acknowledgements

I would like to thank Dr. Lolita Piersimoni, Nicolette Ognjanovski, Dr. Raji Janakiraman, and Valerie Forsyth for their significant contributions to the story presented here. Thanks specifically to Dr. Piersimoni for her work shown in figures 2.1B, 2.4B, and 2.5, and her constant encouragement and support; to Nicolette for creating puupΔlinker, and her work shown in figure 2.4, to Valerie for her work shown in figure 2.9, and to Dr. Janakiraman for her work shown in figure 2.7.

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Table 1: Strains and plasmids

Strain or plasmid	Relevant genotype	Source
Strain		
JM4705	WT (BW23115)	
JM6698	Δ*uup*	transduced from Keio into JM4705
JM5731	Δ*bipA*	transduced from Keio into JM4705
JM6865	Δ*rplA*	Keio
ssb113	*ssb113*	Chase *et al.*, 1984
Δ75*topA*	Δ75*topA*	Stupina and Wang, 2004
Plasmid		
pSG25	wild type *lacZ* reporter plasmid	O'Connor *et al.,* 1997
pSG431	*lacZ* with GUG initiation codon	""
pSG414	*lacZ* with UUG initiation codon	""
pSG413	*lacZ* with CUG initiation codon	""
pSG415	*lacZ* with AUC initiation codon	""
pSG416	*lacZ* with AUA initiation codon	""
pSG417	*lacZ* with AUU initiation codon	""
pSG12-6	*lacZ* with premature UAG stop codon, GUU UAG GCC	""
pSG163	*lacZ* with premature UAG stop codon, CUU UAG CUA	""
pSG627	*lacZ* with premature UAA stop codon, GAA UAA GUU	""
pSG853	*lacZ* with premature UAA stop codon, GUC UAA GUU	""
pSG34-11	*lacZ* with premature UGA stop codon, GUG, UGA GCC	""
pSG3/4	*lacZ* with premature UGA stop codon, CUU UGA CUA	""
pSG12DP	*lacZ* with -1 frameshift	""
pSGlac7	*lacZ* with +1 frameshift	""
pCA24N	Aska empty vector (EV)	Aska Collection
pCA24N*bipA*	multi-copy plasmid, (*bipA*)	Aska Collection
pCA24N*fusA*	multi-copy plasmid, (*fusA*)	Aska Collection
pCA24N*lepA*	multi-copy plasmid, (*lepA*)	Aska Collection
pCA24N*yheS*	multi-copy plasmid, (*yheS*)	Aska Collection
pCA24N*ettA*	multi-copy plasmid, (*ettA*)	Aska Collection
pCA24N*bipA*	multi-copy plasmid, (*bipA*)	Aska Collection
pCA24N*uup*	multi-copy plasmid, (*uup*)	Aska Collection
pCA24N*uup1*	multi-copy plasmid, (*uup,* UupD181N)	this work
pCA24N*uup2*	multi-copy plasmid, (*uup,* UupD465N)	this work
pCA24N*uup1,2*	multi-copy plasmid, (*uup,* UupD181N/D465N)	this work
pCA24N*uupΔCTD*	multi-copy plasmid, (*uup,* UupV550STOP)	this work
pCA24N*uupΔlinker*	multi-copy plasmid, (*uup,* UupΔaa294-300)	this work
pBAD18	pBAD18 empty vector	Murat *et al.*, 2008
pBAD18*uup*	inducible plasmid (*uup*)	Murat *et al.*, 2008
pBAD18*uup1*	low-copy plasmid, (*uup,* UupD181N)	Murat *et al.,* 2008
pBAD18*uup2*	low-copy plasmid, (*uup,* UupD465N)	Murat *et al.,* 2008
pBAD18*uup1,2*	low-copy plasmid, (*uup,* UupD181N/D465N)	Murat *et al.,* 2008
pBAD18*uupΔCTD*	low-copy plasmid, (*uup,* UupV550STOP)	Murat *et al.,* 2008

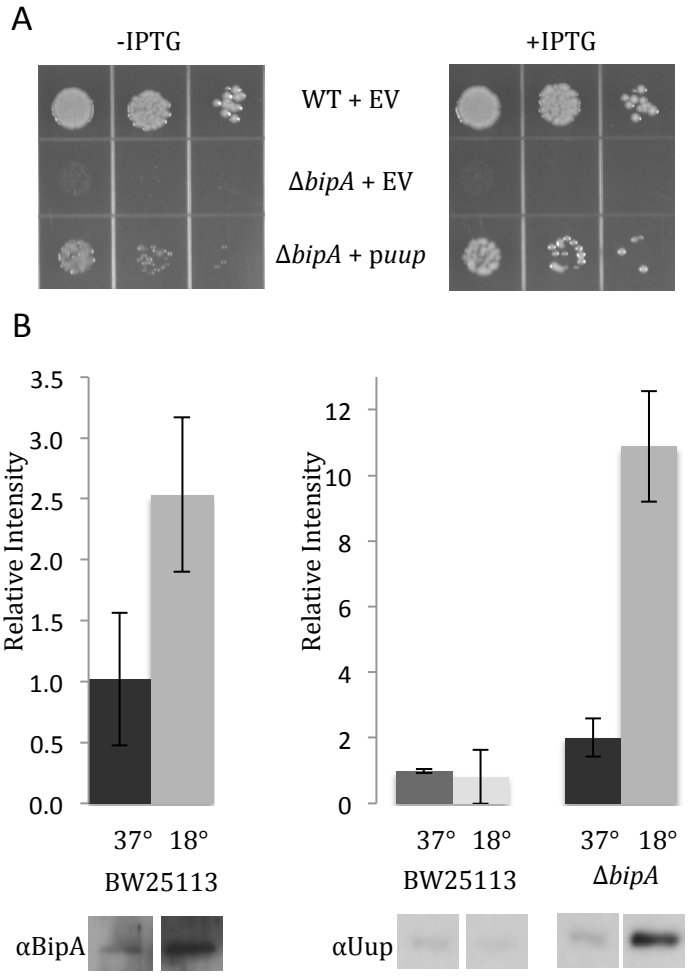

Fig. 2.1: Uup is a Δ*bipA* suppressor. (A) Ten-fold serial deletions showing the growth at 18°C of wild type and Δ*bipA* cells harboring pCA24N (EV) or pCA24N-*uup* (p*uup*) were spotted on LB +/- 25 μM IPTG. (B) Endogenous levels of BipA (left panel) and Uup (right panels) assayed by western blot. Protein levels were examined in wild type (BW25113) or Δ*bipA* mutants grown at either 18°C or 37°C , as indicated. ImageJ scans of 3 replicates were were quantified and averaged (top panel). Error bars show standard deviation. Representative western blots are shown (lower panels).

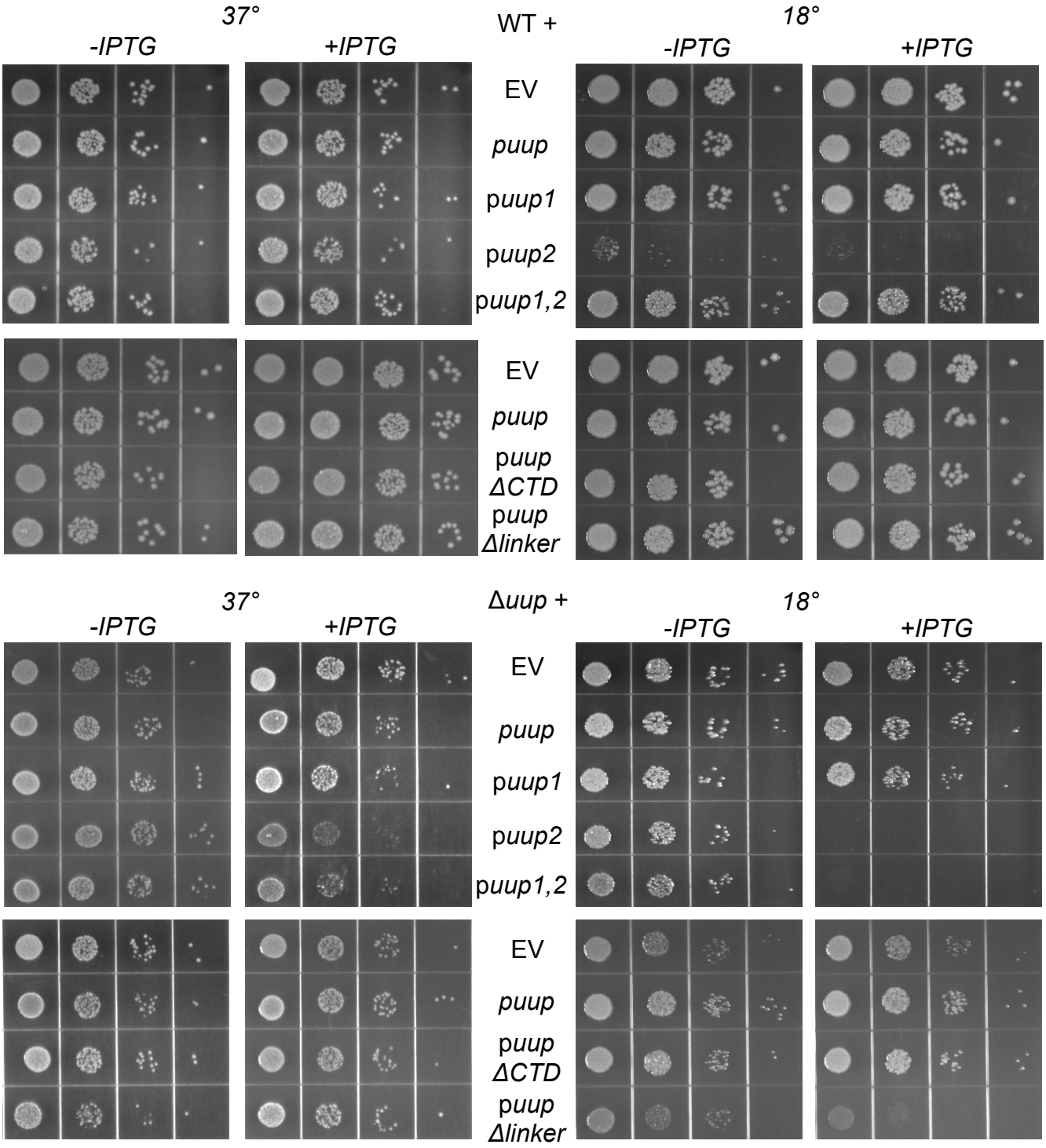

Fig. 2.2: Growth consequences of high-copy Uup variants in presence and absence of endogenous Uup. BW25113 **s**trains carrying Uup or Uup mutant over-expression plasmids in a wild type (top panels) or Δ*bipA* (bottom panels) background were serially diluted and spotted on media with and without 25 μM of IPTG and incubated at 37° or 18° as indicated.

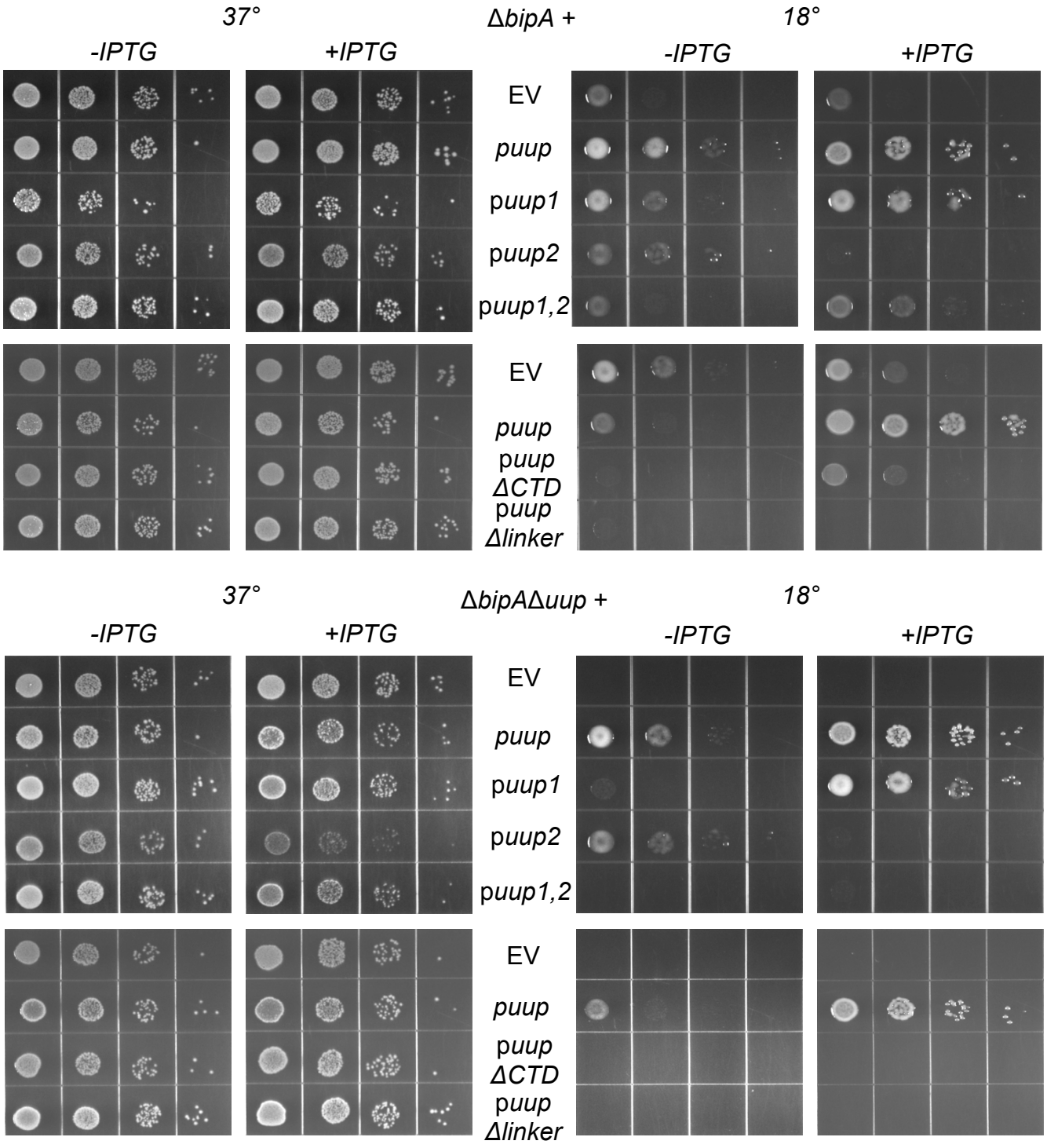

Fig. 2.3: Growth consequences high-copy Uup variants on Δ*bipA* strains. Strains with plasmid-borne Uup variants in a Δ*bipA* (top panels) or Δ*bipA*Δ*uup* (bottom panels) background were serially diluted and spotted on media with and without 25 μM of inducer (IPTG) and incubated at 37° or 18° as indicated.

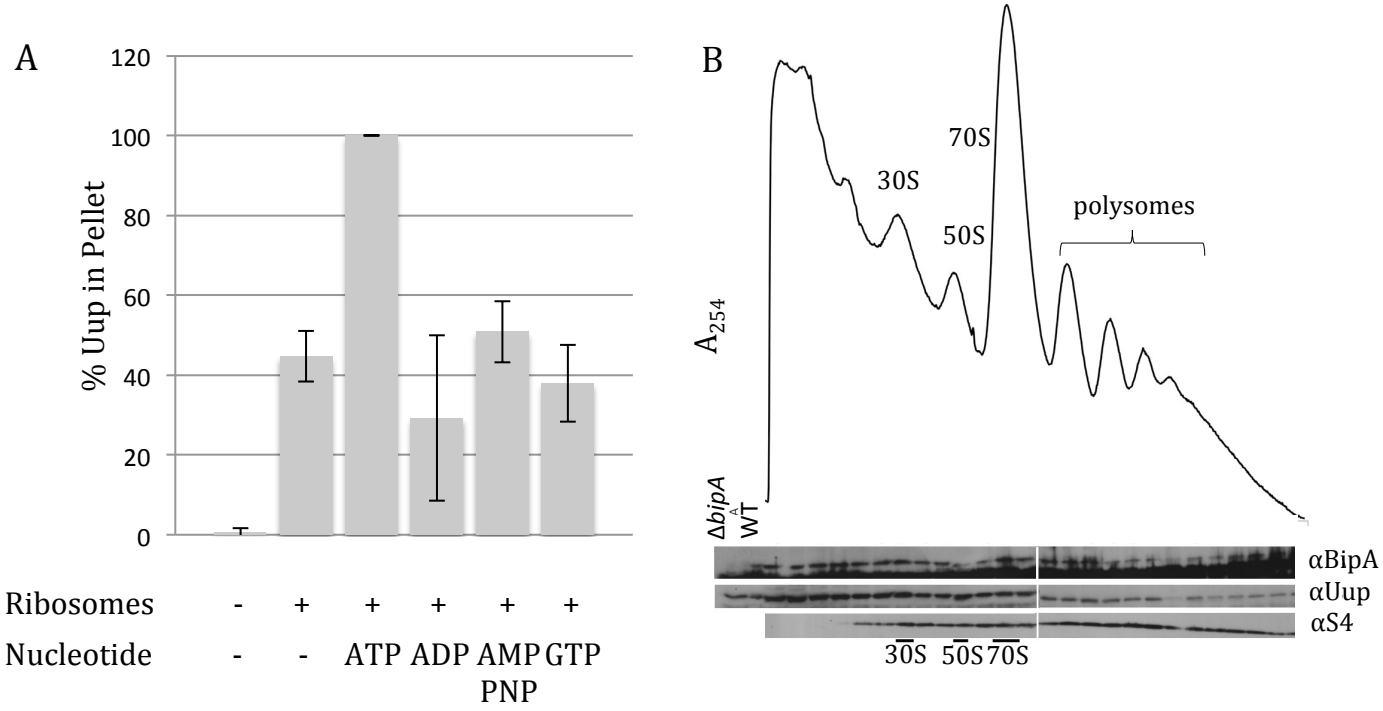

Fig. 2.4: Uup is found on ribosomes. (A) Purified Uup was combined with or without purified ribosomes and ribosomes were pelleted in the presence or absence of various nucleotides, as indicated. The Uup content of pellet and supernatant fractions was quantified by western blotting, and the proportion of the total Uup signal that was found in the pellet for each condition is represented here. Error shows the standard deviation of three replicates. (B) Ribosomes from cells grown at 37°C were separated by sucrose density centrifugation and monitored by absorbance at 254 nm. The position of ribosomes or ribosomal subunits is indicated. Fractions were precipitated with TCA and the positions of BipA, Uup, and ribosomal protein S4 were detected by immunoblot. Also loaded on gel were cell extracts from Δ*bipA* and wild type cells, as indicated.

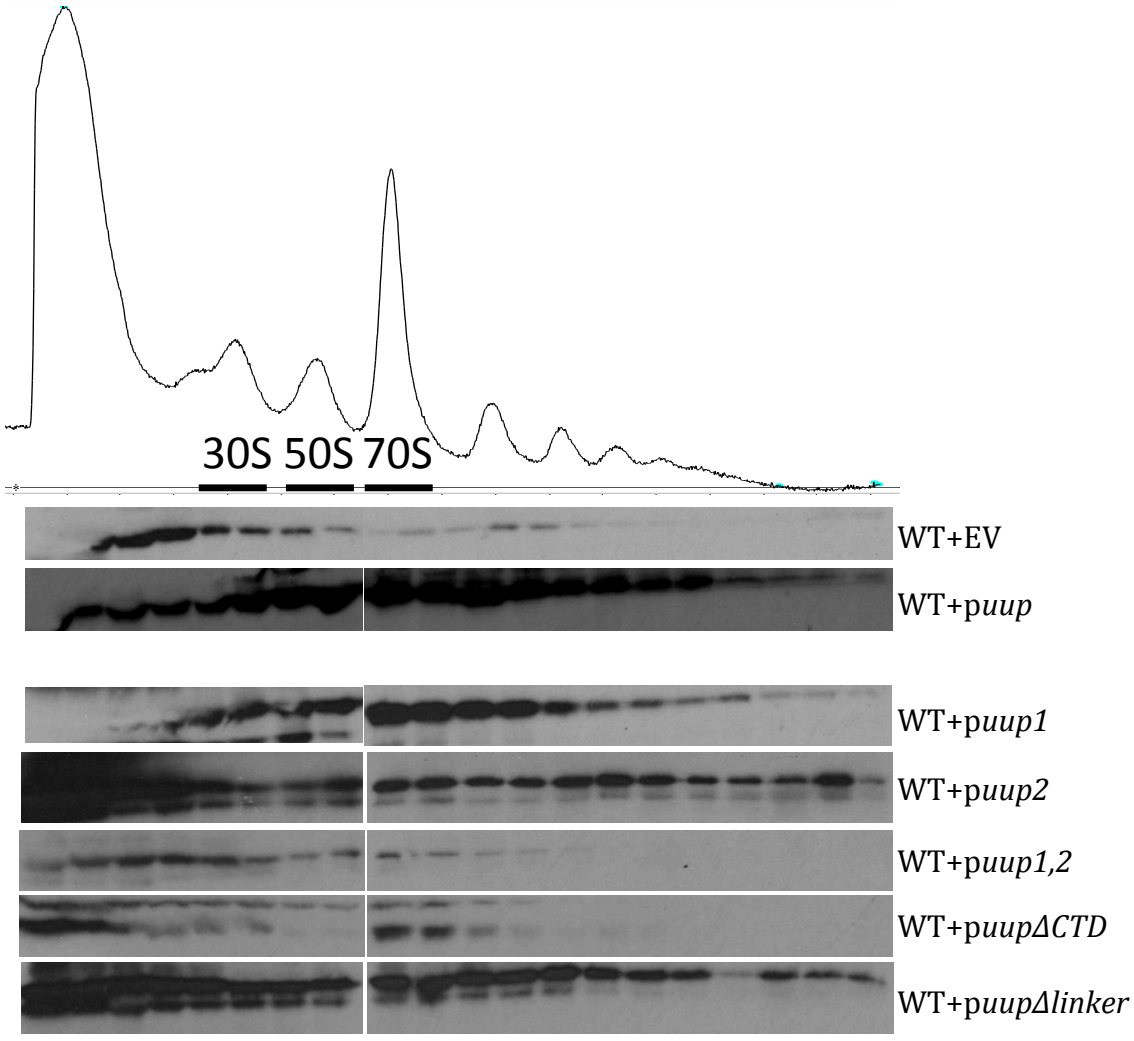

Fig. 2.5: Dominant negative Uup variants associate with ribosomes more strongly than other variants Ribosomes from the indicated strains grown at 37°C in the presence of 25 μM IPTG were separated by sucrose density centrifugation and monitored by absorbance at 254 nm. The position of ribosomes or ribosomal subunits is indicated. Fractions were precipitated with TCA and the positions of Uup and indicated Uup variants were detected by immunoblot (top band where multiple bands are present).

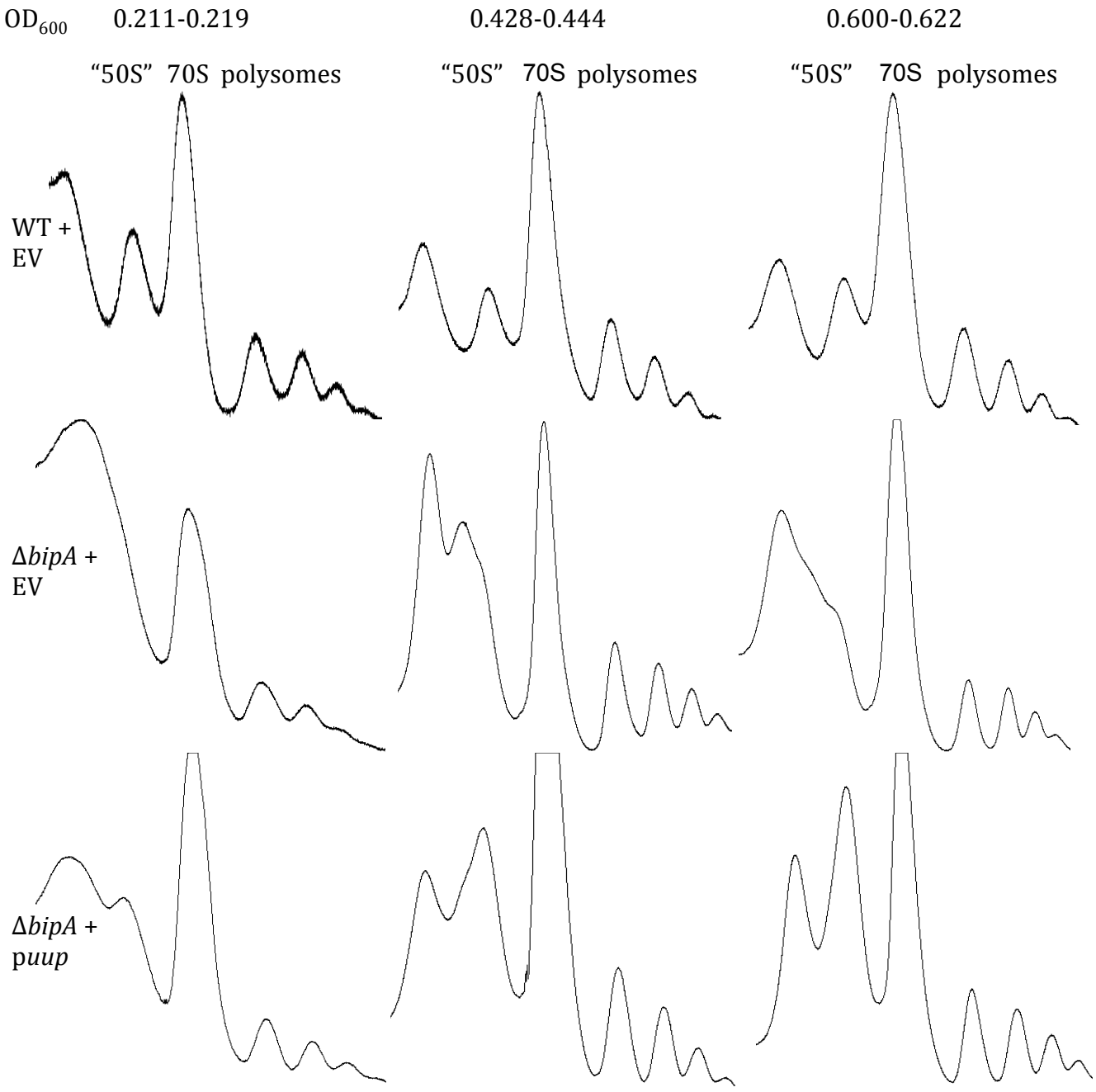

Fig. 2.6: Overexpression of Uup results in earlier correction of growth-phase dependent Δ*bipA* 18° ribosome profile defect. Cultures of WT with empty vector, Δ*bipA* with empty vector, or Δ*bipA* with p*uup* were grown in LB-chlor at 37°C to the indicated OD_{600}s and cells lysed. Ribosomes were separated by sucrose gradient centrifugation on 7%-47% gradients of cell extracts from OD_{600}-matched cultures of the listed strains and followed by absorbance at 245 nm. OD-matched profiles of each strain were aligned by aligning the 70S and 2-mer (1st polysome) peaks.

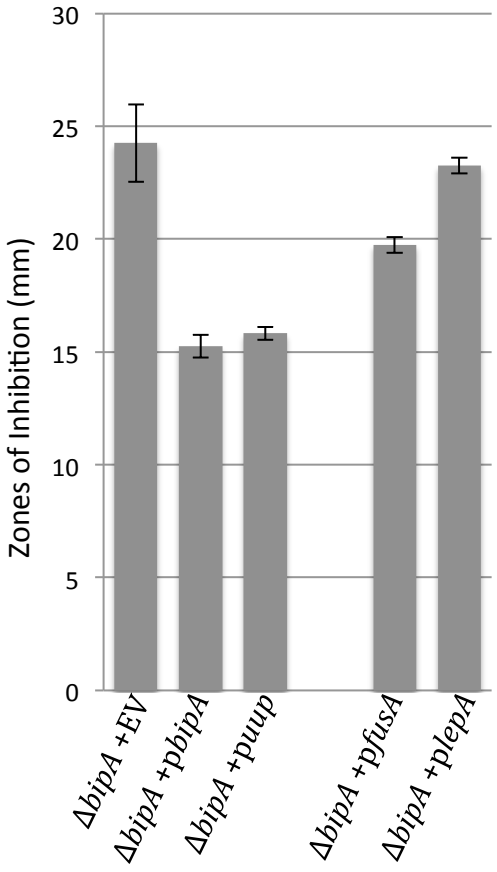

Fig. 2.7: Uup and EF-G suppress Δ*bipA* rifampicin sensitivity. Δ*bipA* cells containing pCA24N (EV) or plasmid-borne *bipA*, *uup*, *fusA* (encoding EF-G), or *lepA* were spread on LB plates containing 25 μM IPTG. Rifampicin disks (5 mg) placed on the plates and the plates were incubated at 18°C. Resulting zones of inhibition were measured for 3-4 samples and error is reported as standard deviation.

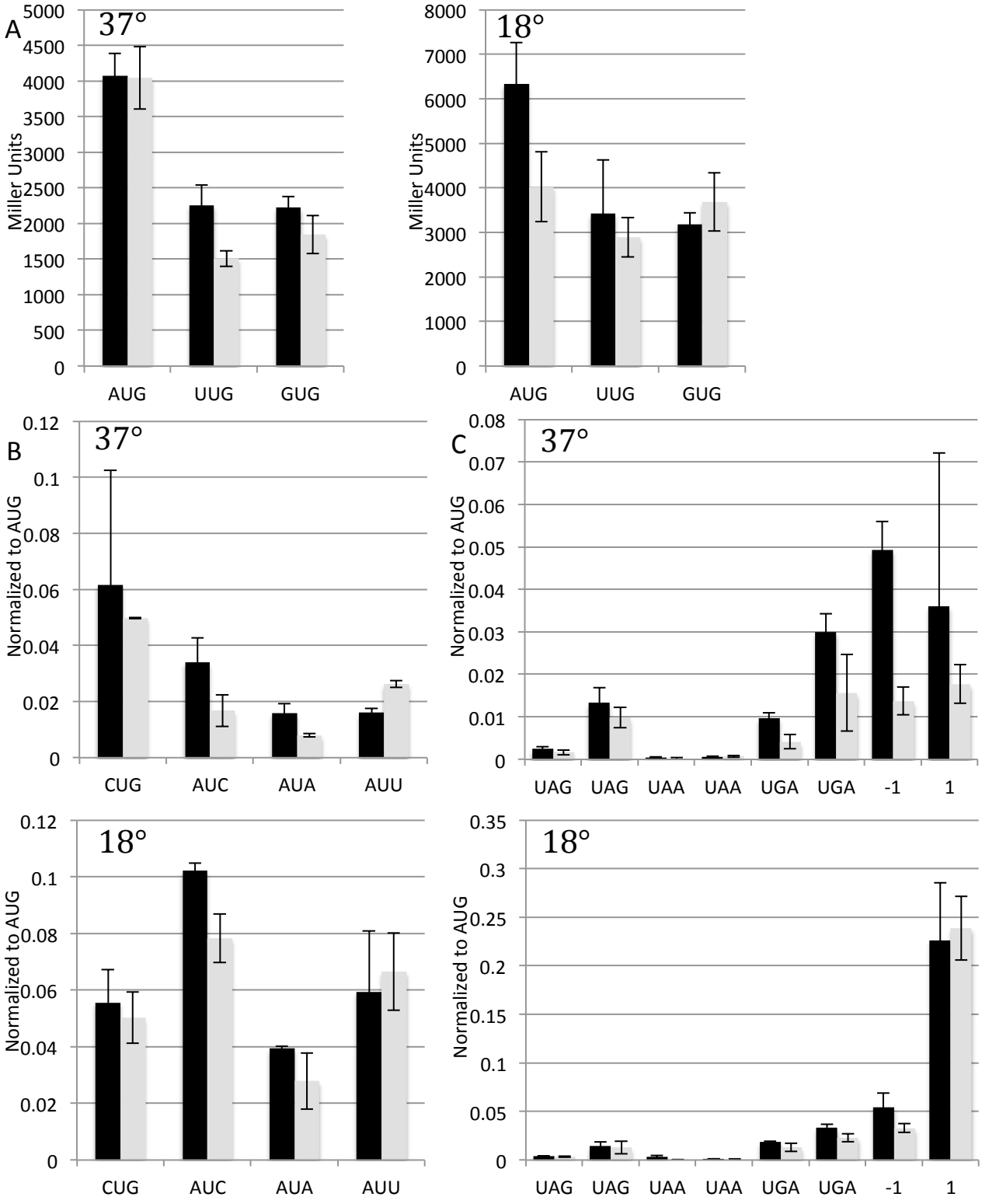

Fig. 2.8: Translation fidelity in a Δ*bipA* mutant. β-galactosidase activity resulting from translation of wild type or mutant *lacZ* reporter plasmids is reported as Miller units. Initiation from start (A) and non-start (B) codons as well as translation through stop codons and +1/-1 reading frame shifts (C) are shown for Δ*bipA* cells ( ) and wild type cells( ). Data represent the average of at least three replicates and error is shown in standard deviation.

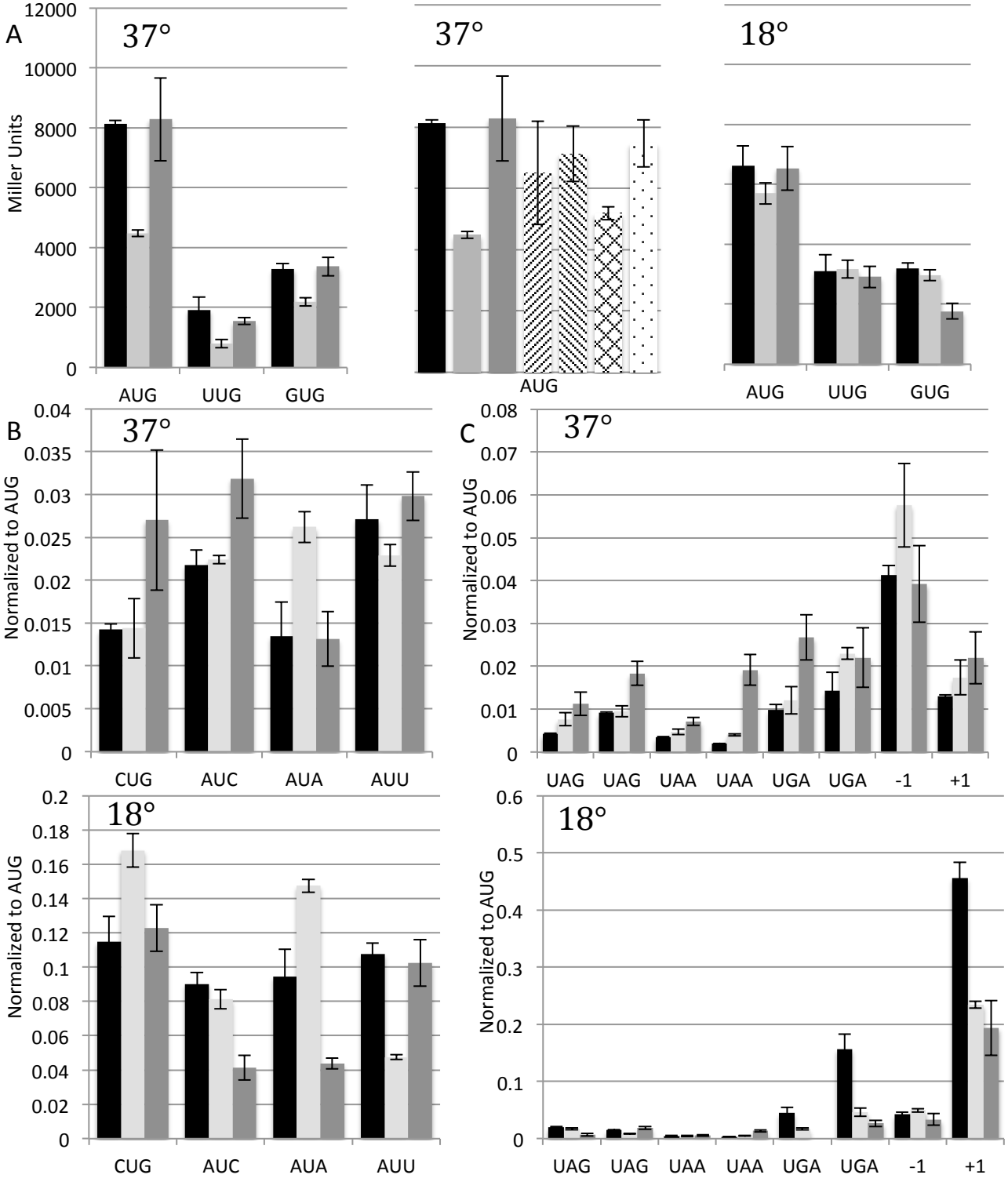

Fig. 2.9: Translation fidelity in a Δ*uup* mutant. β-galactosidase activity resulting from translation of wild type or mutant *lacZ* reporter plasmids is reported as Miller units. Initiation from start (A) and non-start (B) codons as well as translation through stop codons and +1/-1 reading frame shifts (C) are shown for Δ*uup* + EV ( ), Δ*uup* + p*uup* ( ) and wild type + EV cells( ). The effect of induction (25 μM IPTG) of plasmid-borne mutant *uup* (p*uup1* ( ), p*uup2* ( ), p*uup1,2* ( ), or p*uupΔCTD* ( )) are also shown (A). Data represent the average of at least three replicates and error is shown in standard deviation.

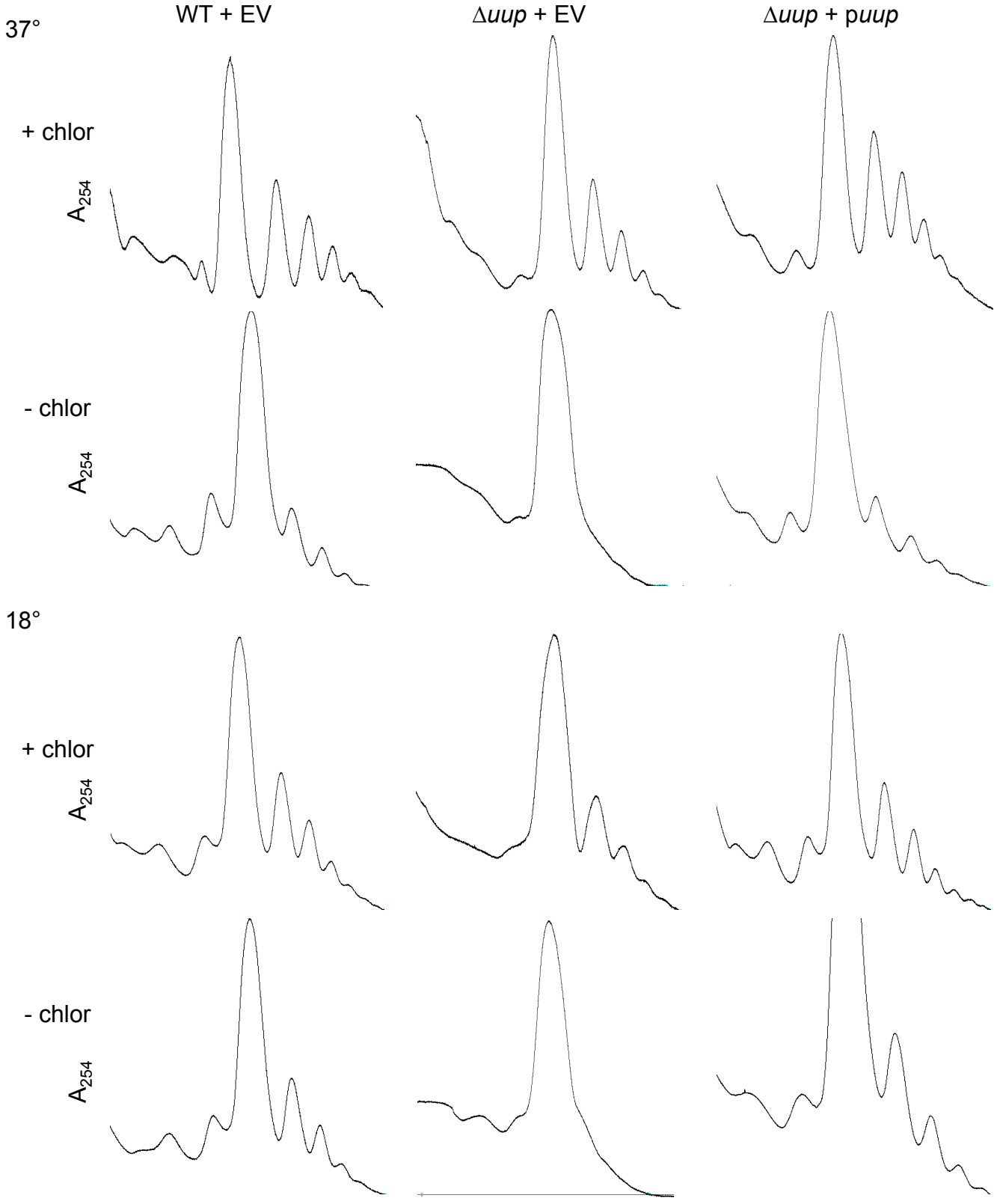

Fig. 2.10: Deletion of Uup results in decreased polysomes. Cell extracts from BW25113 (WT) + pCA24N (EV), Δ*uup* + EV, and Δ*uup* + pCA24N*uup* (p*uup)* grown at 37° and 18° (as indicated) to mid log were separated by centrifugation of clarified cell lysate over 4% to 47% sucrose density gradients in the presence or absence of 0.2 mg/ml chloramphenicol (chlor) and assayed for absorbance at 254 nm.

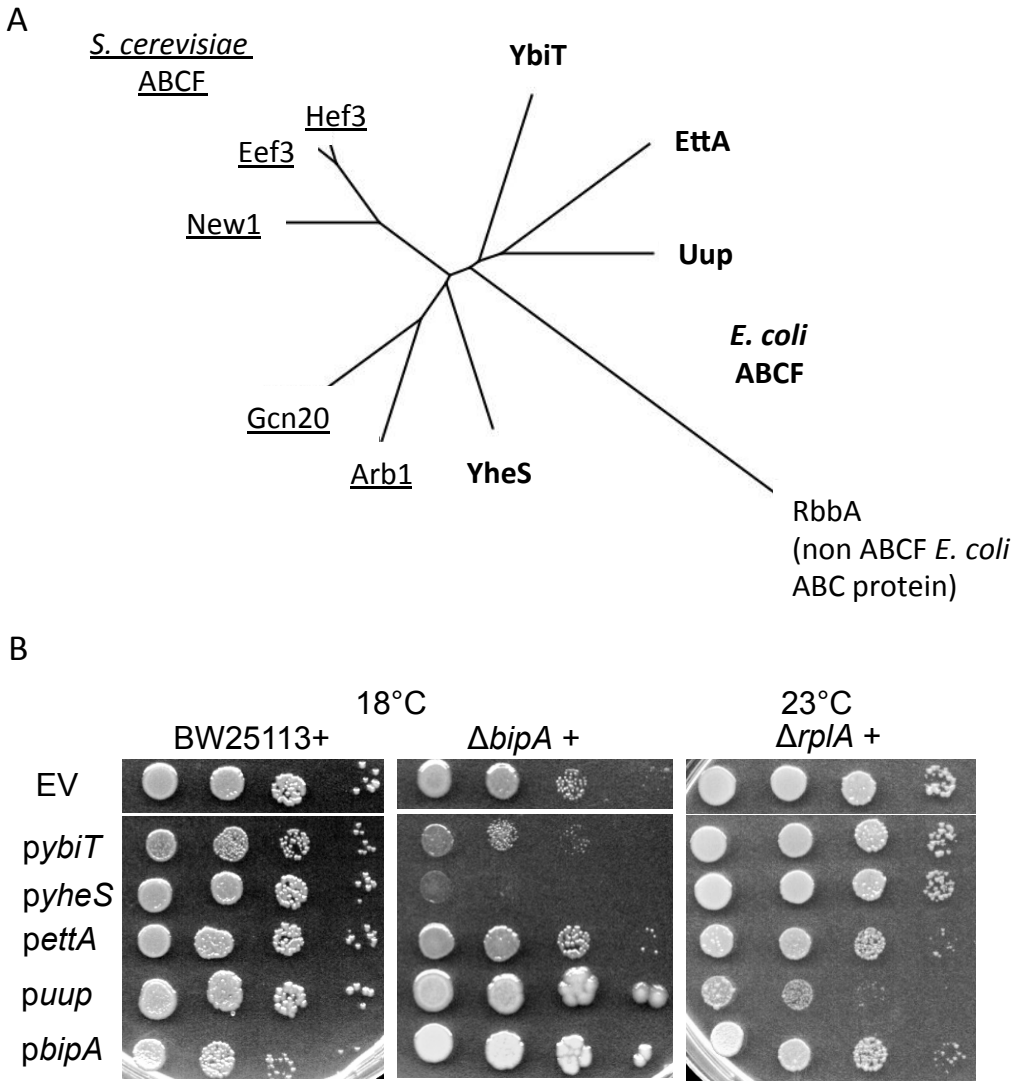

Fig. 2.11: Uup and EttA are similar (A) Phylogenetic tree based on the sequences of the ABCF proteins of *E. coli* (**bold**) and *S. cerevisiae* (underlined) with RbbA, a non-ABCF, ribosome-associated, *E. coli* ABC protein, as out-group. (B) Serial dilutions of cultures carrying the ABCF proteins of *E. coli* on IPTG-inducible plasmids in a wild type, Δ*bipA* or Δ*rplA* (L1) background were spotted on media containing 25μM IPTG and incubated at cool temperatures (18° or 23°C, as indicated).

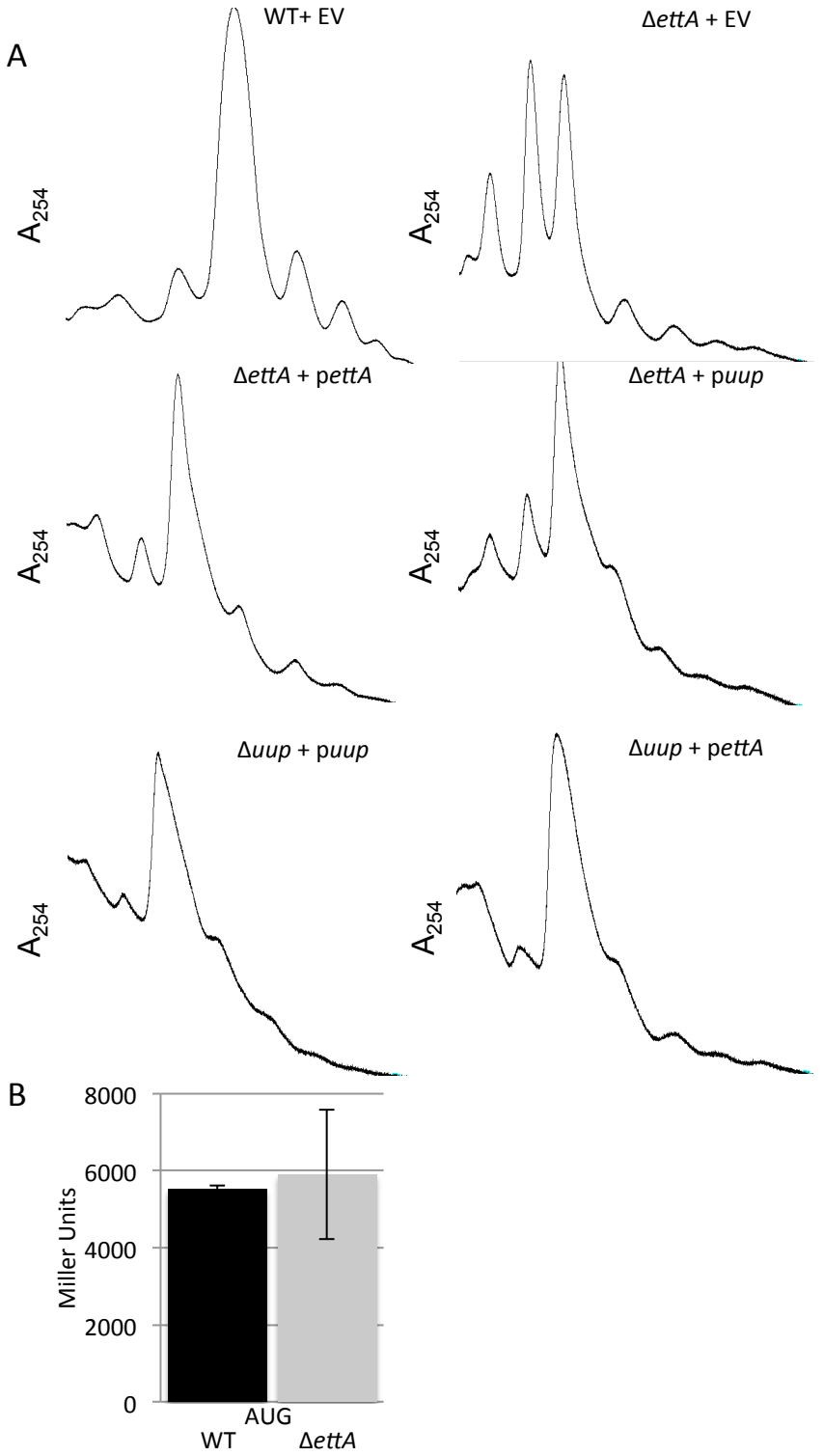

Fig. 2.12: While mutants of *uup* and *ettA* have different phenotypes, high-copy Uup and EttA suppress each other's ribosome profile defects. (A) Cell extracts from BW25113 (WT) + pCA24N (EV), Δ*ettA* + EV, Δ*ettA* + pCA24N*ettA* (p*ettA*), Δ*ettA* + pCA24N*uup* (p*uup*), Δ*uup* + p*uup*, and Δ*uup* + p*ettA* grown at 37°C to mid log were separated by centrifugation of clarified cell lysate over frozen and thawed 23% sucrose density gradients in the absence of chloramphenicol (chlor) and assayed for absorbance at 254nm. (B) β-galactosidase activity resulting from translation of AUG-start *lacZ* reporter plasmid is reported as Miller units. Data represents average of three replicates. Error is shown as standard deviation.

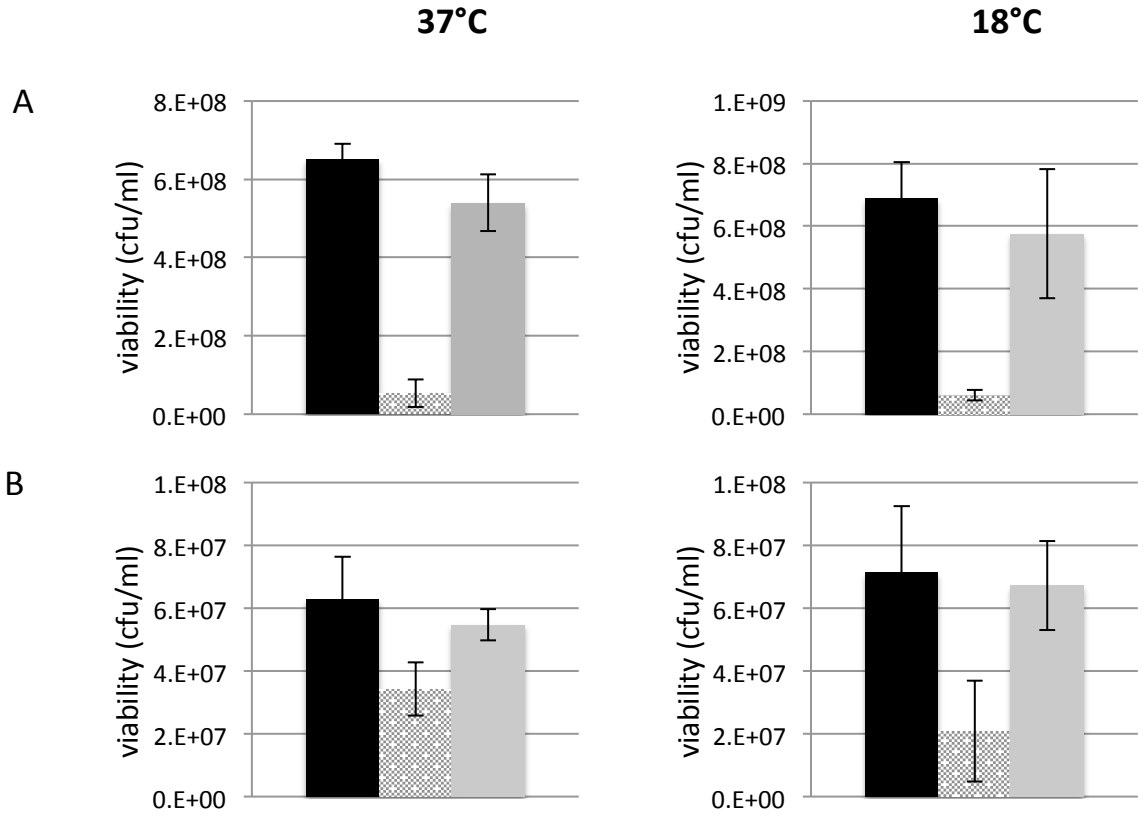

Fig. 2.13: Overexpression, but not loss, of Uup reduces viability. WT+EV ( ), WT + p*uup* ( ), and Δ*uup* + EV ( ) overnight cultures were diluted 1:100 in LB chlor and grown to an OD_{600} above 0.75 (A) or ~0.4 (B) in the presence of 25 μM IPTG. Equivalent OD_{600} units were plated onto media containing 25 μM IPTG and the cfu/ml were calculated for each strain.

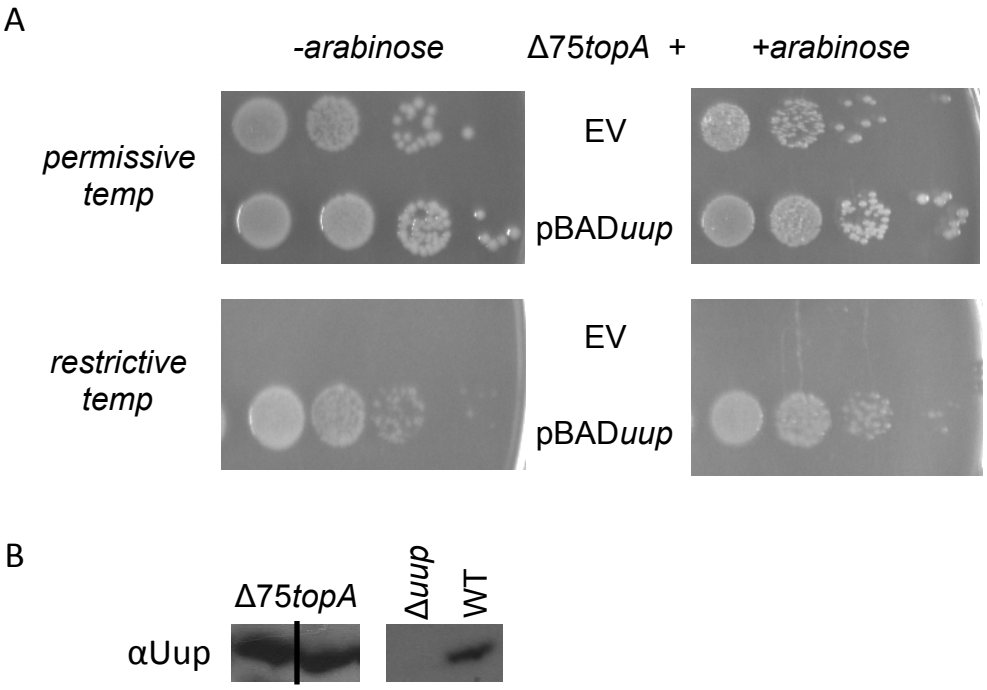

Fig. 2.14: Uup may be important for translation of *topA*. Temperature-sensitive (A) *topA*Δ75 cultures carrying EV or pBAD18*uup* were serially diluted and spotted on media with and without 0.02% arabinose and incubated at the permissive (37°C and 42°C, respectively) and restrictive (42°C and 37°C, respectively) temperatures. (B) OD_{600}-normalized cell extracts of *topA*Δ75 (two replicates), Δ*uup,* and wild type cells grown to mid-log OD_{600} and analyzed by western blot for levels of Uup.

A

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YLLEKEEALRVEELQNAEFDRKLAQEEVWIRQGIKARRTRNEGRVRALKAMR----RERGERREV
:|.:|::.|..|..|.|...:.:.:|..|:|||.|.|:::.:.|:...:.:.    ::|.|..|:
WLEQKDQRLAQEASQEAARRKSIEKELEWVRQGTKGRQSKGKARLARFEELNSTEYQKRNETNEL
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B

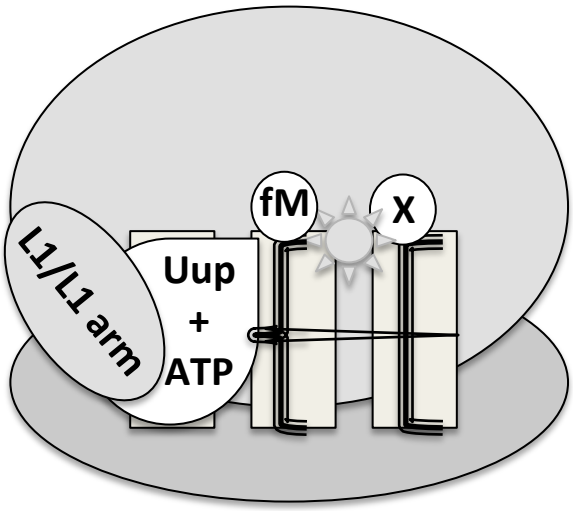

Fig. 2.15: Uup may also be an E-site translation factor. (A) Uup (top) is >26% identical and >53% similar to EttA (bottom) through the P-site tRNA interacting Motif. (B) I propose that Uup, in complex with ATP, binds in the E-site of the ribosome, making contacts with L1/the L1 arm and the $tRNA^{fMet}$ (based on conserved residues in Uup and EttA), and promotes formation of the first peptide bond.

Chapter 3

Characterizationof a Δ*rplA* mutant of *E. coli*

ABSTRACT

The ribosomal protein L1 (encoded by *rplA*) and the flexible rRNA arm to which it binds make important contributions to the process of translation. However, little is known about the effects of loss of L1 on ribosome function. Here we show that loss of L1 affects the specificity of both the A and P sites of the ribosome. We also show that a strain lacking L1 produces an aberrant ribosome profile with an accumulation of 50S ribosome subunits. Finally, we present three major classes of high-copy suppressors of the slow growth in the cold of a ΔL1 strain. The largest of these classes are proteins acting up and down-stream of RpoS. We also find proteins able to affect ribosome makeup in the area around the A and P-sites. The smallest class of suppressors of ΔL1 slow growth in the cold are involved in DNA replication. Most of the slow-growth suppressors tested do not suppress the ΔL1 ribosome defect. To our knowledge, this is the first inspection of a "modern" L1-deleted strain, constructed in a clean background and not collected as a spontaneous suppressor of another lesion.

INTRODUCTION

The bacterial ribosome consists of enzymatically-active ribosomal RNA (rRNA) and many associated proteins. While most of the ribosome is a tightly-packed and somewhat immobile mass of proteins and rRNAs, there are two protuberences on the 50S subunit that are quite flexible. The shorter of these protuberences extends from the ribosome near the E-site [1, 2]. It is called the L1 stalk, and is comprised of flexible rRNA bound by ribosomal protein L1 [1-3].

The protein L1 contains 233 amino acids, and has a high Lys/Arg ratio [4]. L1 is highly conserved throughout the domains of life [5]. Structurally, L1 is comprised of two domains. The larger domain, domain I, is interrupted by the sequence of the smaller domain II [3]. Both domains form globular structures that rotate around the "hinge" region that connects them. The interface of the two domains forms the rRNA binding region of L1, and the native accessibility of this region strongly influences the strength of L1-RNA binding [3]. Domain I of L1 makes the majority of contacts with the rRNA [2, 3, 6]. Domain I interacts primarily with helix 77 of the L1 arm, including the conserved residues of the rRNA [2, 3]. Domain II makes fewer contacts with the rRNA, primarily in the interconnecting loop region between nucleotides G2165 and A2171 [2, 3].

Early work with L1 deletion strains obtained as spontaneous revertants of kasugamycin and spectinomycin dependant mutants showed slow growth, a marked decrease in protein synthesis (most likely at the step of elongation), and a defect in tRNA binding [7-9]. Further work in the same mutants demonstrated that the tRNA binding effect was specific to amino-acylated tRNA binding initially in the P-site. The reduction in protein synthesis was explained by a decrease in the ribosome-stimulated GTPase activity of EF-G on these ΔL1 mutants [9].

L1 and the L1 arm perform important functions during translation. The L1 arm is ideally situated to interact with tRNA in the E-site and control E-site occupancy in coordination with tRNA movement through the A and P sites [2]. The L1 arm adopts a closed position upon EF-G binding and makes contacts with the P-site tRNA that are maintained as the P-site tRNA transits to the E-site [10-12]. This coordination between L1/the L1 arm and EF-G may be the basis for the decrease in EF-G activity on ΔL1 ribosomes mentioned earlier [9].

A number of proteins have been shown or proposed to interact with L1 as part of their activity on the ribosome. These include the essential yeast protein eEF3, which is proposed to modulate the position of the L1 arm and regulate E-site occupancy during translation in yeast [13-15], and the bacterial proteins EF-P and EttA [16-18]. EF-P makes contacts with both L1 and the P-site tRNA, and is necessary for the efficient translation of stall-prone poly-proline runs [16, 19-22]. EttA also binds L1, the L1 arm, and the P-site tRNA, including certain characteristics

unique to the initiator $tRNA^{fMet}$, and is able to either inhibit or promote formation of the first peptide bond of protein synthesis, depending on the level of cellular energy [17, 18].

Cross-talk between the E-site of the ribosome, bounded by the L1 arm, and the A-site, poises L1 to be able to make both specific and general contributions to translation [14, 23, 24]. Coordinated action of the L1 arm during translocation, coupled with contacts with both the P-site and E-site tRNAs raise the potential for L1 to play an active role in protein synthesis. Here, we further explore the effects of loss of L1 on protein synthesis, and present 3 major classes of proteins that are high-copy suppressors of ΔL1 slow growth in the cold.

METHODS

Bacterial strains, culture conditions, and growth measurements

E. coli strains and plasmids used are listed in table 1 and table S1. Cultures were grown at the indicated temperatures in Luria-Bertani (LB) broth (10 g tryptone, 5 g yeast extract, 10 g NaCl per liter) or on LB agar plates (LB broth plus 1.5% agar) with antibiotics as appropriate. Culture growth was monitored by measuring the absorption at 600 nm. Antibiotics were used at the following concentrations: 20 μg/ml chloramphenicol (chlor), 30 μg/ml kanamycin (kan), 12.5 μg/ml tetracycline (tet).

Isolation of suppressors

Δ*rplA* competent cells (Keio collection) were transformed with a library of Aska plasmids, plated on LB chlor plates with 25 μM IPTG, and incubated at 18°C overnight to allow distinguishable colonies to form. Transformants with larger colony sizes than the majority of colonies were isolated, and their plasmid inserts sequenced. Putative suppressor plasmids were then confirmed by independent isolation from the appropriate Aska strains, transformation into Δ*rplA* competent cells, serial dilution, and plating on LB chlor 25 μM IPTG (incubated at 18°C), with growth compared to serial dilutions of Δ*rplA* cells containing empty vector (pCA24N).

Plate growth assay

Overnight cultures were serially diluted 1:10 in 1 ml sterile LB media 7 times. The first dilution was discarded, and 5 μl samples of dilutions 2-6 were spotted onto LB-agar plates with the appropriate antibiotics with and without 25 μM IPTG. Plates were incubated at the temperature indicated until the complemented strains reached a consistent size. Pairs of plates containing the same dilutions with and without IPTG were incubated and removed together.

Ribosome Profiles

Cultures were grown at 37°C or 18°C until they reached an OD_{600} of 0.350-0.800. Cells were pelleted and prepared for sucrose density gradient centrifugation as previously described [25]. Approximately 13 OD_{260} units were loaded on top of 4.95 ml 7%-47% sucrose digital gradients. Loaded gradients were centrifuged at 41000

rpm for 1.5 h in a Beckman SW50.1 rotor. After centrifugation, gradients were fractionated as in [26] using a flow rate of .350 ml/min for the 4.95 ml gradients Profile data was captured digitally on a computer connected to the ISCO UA-5 detector, or as in [26].

RESULTS

In light of the growing evidence for a vital role in translation for ribosomal protein L1 (encoded by *rplA*) [5, 9, 16-18, 27, 28], we asked how translation differs in the absence of this protein. We measured β-galactosidase activity in wild type and ΔL1 cells carrying one of a suite of *lacZ* reporter plasmids (Fig. 3.1). These plasmids provide information on A-site fidelity by assaying initiation with $tRNA^{fMet}$ from various start and non-start codons [29]. P-site fidelity is assayed by measuring functional protein produced as a result of read-through of introduced stop codons [29]. Frame-shifting events are also measured [29]. Because the L1 mutant grows very slowly in the cold, and is prone to spontaneous suppressors, the results are shown only for growth at 37°. The results at 18°C are very similar for the cases tested with the exception of a vastly increased +1 frameshift activity in the cold.

L1 has been reported to help stabilize the initiator tRNA in the P-site during initiation of translation, and proteins have been shown or suggested to interact both with the initiator tRNA and the L1 protein and thereby affect initiation [17, 18]. Initiation with fMet tRNA from non-AUG start codons requires codon-anti-codon mispairing at the P-site of the initiating ribosome. The level of initiation from non-

AUG codons is taken as a measure of the error-proneness of the P-site. We found that translation from the primary start codon, AUG, is increased by about 2x in the mutant compared to the wild type (Fig. 3.1A). However, translation from the alternate start codon GUG decreased almost 100% in the Δ*rplA* mutant. GUG is used as the start codon in 7% to 14% of *E. coli* genes [30, 31]. It is possible that some of the increased translation we see for other "start" codons is due to increased availability of ribosomes because of the sharp decrease in translation from GUG. Translation from UUG was equivalent in the wild type and mutant strain. Minimal effect on translation from non-start codons is seen (Fig. 3.1B) in the absence of L1.

The loss of L1 could make the ribosome more or less sensitive to ATPase translation factors shown or suspected to interact with L1 and the initiator tRNA. Deregulation of L1-interacting E-site ATPase factor activity could reduce a stabilizing effect needed for translation from GUG, and possibly also reduce a discriminating effect that prevents initiation from the non-start codons tested. The increased translation from the AUG could be partially the result of increased ribosomes available that would usually be translating GUG-start messages. Alternately, normal translation from AUG, like the non-start codons, could be limited by a discriminatory action involving L1.

Stop codon read-through is taken as a measure of A-site specificity. We see little effect on stop codon read-through in the absence of L1 (Fig. 3.1C). There is slightly more read-through of one UGA stop codon in the absence of L1 than in wild type

cells. However, changing the genetic context of the UGA restores stop codon function. The stop codon read-through seen could be due to defects in long-range communication between the L1-bounded E-site and the A-site, but seem more likely to be tied to local effects of the plasmid sequence.

Frame-shifting in the -1 direction is increased in the ΔL1 mutant. The -1 frameshift is likely due to movement of the tRNA in the P-site relative to the mRNA, while the +1 frame shift probably involves out-of-frame binding in the A-site due to ribosomal pausing [29]. At 18°C, there is a striking increase in the level of +1 frame-shifting in the wild type strains and an even greater increase in the ΔL1 mutant (data not shown). It is possible that the cold temperature produces a general slowing of ribosomal motion that amplifies the effect of the stop-codon induced pause.

We next asked whether the discrimination errors reported above were accompanied by any visible defect in the ribosome profile of the mutant strain. Cultures grown at both 37° and 18° showed a striking increase in 50S subunits compared to 70S holo-ribosomes (Fig. 3.2), suggesting either a decrease in ribosome coupling or an imbalance in ribosomal subunit translation or assembly. We did not, however, observe a secondary peak that would indicate significant quantities of immature 50S particles. While all ΔL1 strains showed a 50S accumulation, the apparent severity of the defect varied from culture to culture, even when prepared and assayed simultaneously. This variability was independent of growth temperature and harvest OD_{600}.

Slow growth in the cold is a common phenotype of ribosome-associated mutations. The ΔL1 mutation also results in severe slow growth in the cold, as well as slower growth at 37°. A suppressor screen to find genes that would increase growth rate of the mutant in the cold when induced ectopically was carried out by transforming competent ΔL1 with an Aska plasmid library [32] and isolating colonies that grew faster than the parent strain at 18°. Plasmids from these faster-growing colonies were isolated and sequenced. Plasmids containing putative high-copy suppressing genes were then independently purified from the Aska collection and used to transform the ΔL1 background. Growth of the transformants was assayed in the cold to confirm suppression (Fig. 3.3).

Upon inspection, most of the isolated suppressors of the ΔL1 slow growth in the cold phenotype with known functions could be separated into one of three functional groups. These groups are: ribosome-associated factors, DNA replication factors, and RpoS regulation/regulon proteins.

RpoS regulation/regulon

The largest group of ΔL1 suppressors could be tied to RpoS. We isolated six genes involved in regulating RpoS levels (*gcvA, sspA, greA, greB, iraM, iraD)*, *rpoS* itself, and three RpoS-regulated genes as plasmid-borne high-copy suppressors of ΔL1 slow growth in the cold. We also isolated FrsA, a protein that may result in sequestration

of σ^{70} when over-expressed, thus freeing the core polymerase for association with σ^{S}. The proteins and their relationship to RpoS are diagramed in Fig. 3.4.

In addition to *rpoS* itself, we isolated *iraM* and *iraD*, encoding two of the three structurally unique but functionally similar anti-adaptors that help prevent RpoS degradation in response to magnesium starvation (IraM) [33] and DNA damage and stationary phase (IraD) [33, 34]. The third known RpoS anti-adapter, *iraP,* was not isolated. This could be because of a difference in function and regulation of IraP *in vivo* (IraP responds to a much more specific set of stimuli than the other two anti-adapters [34-36] and interacts uniquely with RssB to prevent RpoS degradation [37]), or because our screen was not saturated, and we did not isolate all possible ΔL1 high-copy suppressors.

We also isolated *greA* and *greB* as ΔL1 suppressors. The two transcription factors encoded by these genes are structurally similar to the transcription factor DksA [38, 39], which most likely binds in the secondary channel of RNA polymerase and helps keep IraD levels low in exponential phase [35, 38, 39]. GreA/B are able to out-compete DksA when present in sufficient concentration [40, 41], thus relieving DksA-mediated IraD regulation resulting in stabilization of RpoS via increased levels of anti-adapter. As transcription factors, GreA/B also have multiple other targets, and could also contribute to the suppression of ΔL1 slow growth in the cold via changes in the levels of other, non RpoS-related, regulated proteins. Indeed,

transcription of the ribosomal RNA operon *rrnB* is stimulated by both GreA and GreB [42].

We also isolated *sspA* as a suppressor. SspA expression increases as the cell enters stationary phase, and is induced by starvation for many key nutrients [43]. Functional RelA is required for the induction of *sspA* and the *sspA* promoter region has similarity to a know ppGpp-responsive promoter, suggesting that endogenous SspA levels are responsive to ppGpp [43, 44]. SspA affects the levels of multiple proteins, including H-NS [43, 45]. Although SspA has been shown to interact functionally with the RNA polymerase [46, 47], it limits H-NS levels post-transcriptionally [45]. H-NS negatively regulates RpoS [48, 49], and SspA has been shown to be important for maintaining normal RpoS levels [45]. Therefore, over-expressing SspA can be expected to increase levels of RpoS by decreasing functional H-NS levels. Curiously, the *sspAB* operon is located immediately downstream of the operon containing *rplM*, another ΔL1 suppressor [43].

GcvA is a regulatory protein that alternately represses and activates transcription of the *gcvTHP* operon, based on cellular needs and nutrient levels [50, 51]. Proteins in the *gcvTHP* operon are responsible for creating methyl groups for use in biosynthetic pathways, including purine synthesis and other cellular methylation reactions [50, 52, 53]. In addition to regulating *gcvTHP* expression, GcvA indirectly increases RpoS levels in the cell via the small RNA GcvB (which is also involved in regulation of a vast network of biosynthestic genes) [54].

The last high-copy suppressor that could affect the level of σ^s activity is FrsA. FrsA interacts with the phosphoenolpyruvate:sugar phosphotransferase system (PTS) by binding to the unphosphorylated form of the phosphocarrier protein IIA^{Glc} [55, 56]. Over-expression of FrsA leads to a shift in the cellular metabolism from respiration to fermentation [55, 56]. However, the PTS also intersects with regulation of the σ^{70}/σ^S competition for core polymerase. In the PTS, phosphate travels from PEP through a series of phosphocarrier proteins. HPr is the protein above the FrsA-binding IIA^{Glc} in the PTS [57]. Under normal circumstances and when glucose is abundant, phosphate moves quickly through the PTS and the phosphocarrier proteins exist mainly in their dephophorylated states. Dephosphorylated HPr is able to bind the anti σ^{70} factor, Rsd, and titrate it away from σ^{70}, freeing σ^{70} to be used in transcription [58]. When glucose levels decrease, phosphate backs up in the PTS leaving the components phosphorylated. The phosphorylated form of HPr is unable to bind Rsd [58], freeing it to sequester σ^{70}, thereby promoting translation from σ^S-dependent promoters in stationary phase. It is conceivable that the excess FrsA produced from our suppressing plasmid titrates out enough dephosphorylated IIA^{Glc} to prevent passage of the phosphate from HPr, disrupting Rsd sequestration and favoring σ^S in the σ^{70}/σ^S competition for the core polymerase.

Although RpoS regulates over 100 known genes [35], we only isolated three of these as high-copy suppressors of ΔL1 slow growth in the cold. As the screen was almost certainly not saturated, it is unlikely that these represent the full compliment of

RpoS-regulated high-copy suppressors of ΔL1 slow growth in the cold. However, it is also possible that other RpoS-regulated suppressors require the coordinated action of two or more proteins, which would not be detectable in a single-gene plasmid library screen.

CsiE, one of the RpoS-induced suppressors of the ΔL1 slow growth in the cold, is induced from its native promoter during the initial transition into stationary phase. Addition of exogenous cAMP after the beginning of stationary phase produces a greater than normal spike in expression. While starvation for nitrogen, phosphate, and carbon all induced CsiE expression, the largest level of induction is seen for carbon starvation and is RelA dependent [59]. CsiE has a higher level of induction during exponential phase than usual for RpoS-induced proteins [59]. Evidence indicates that CsiE is only partially controlled by RpoS, but its other regulators are unknown, as is its function [59].

BcsA, the second RpoS-controlled ΔL1 suppressor, is involved in cellulose biosynthesis [60]. Like CsiE, BcsA is expressed throughout growth, and increases in early stationary phase [59]. BcsA expression is affected both by proteins that work cooperatively with RpoS and by at least one RpoS-independent regulator [61]. High BcsA levels could make colonies appear larger because of higher level of capsule synthesis.

The last member of the RpoS regulon identified as a suppressor of ΔL1 slow growth in the cold is NhaR. NhaR shows RpoS and DsrA dependant up-regulation in the cold [62]. NhaR is also controlled by the acid-responsive protein YdeO and the biofilm repressing protein CsrA [63, 64]. In keeping with its multiple regulators, NhaR in turns regulates multiple proteins of diverse function. NhaR is required for induction of the sodium proton anti-porter NhaA [65] in response to high Na+ concentration [66, 67]. NhaR also regulates the *pgaABCD* operon important for biofilm formation as well as the stress inducible gene *osmC* (which is also regulated independently by RpoS at a different promoter and by RcsB at the same promoter as NhaR action) [68-70].

Ribosome/Translation Connected Suppressors

Another significant group of suppressors of the ΔL1 slow-growth phenotype is proteins that act on or are part of the translation machinery. While only one ribosomal protein (RplM) was isolated as a ΔL1 high-copy suppressor, a variety of ribosome-associated and ribosome maturation factors were found to suppress when induced from a multi-copy plasmid. Figure 3.5 shows the RNA contact regions of the known interactions between the ribosomal and ribosome-associated proteins mentioned in this section onto a depiction of the large subunit rRNA color-coded to depict distance from the peptidyl transferase center (PTC). These sites of contact and connectivity cluster around the PTC and A-loop, suggesting that loss of L1

necessitates compensation in the PTC and A-site, possibly in order to preserve orderly translocation. This is in keeping with the results of our ribosome error assays, which showed loss of specificity at both the A and P sites. This seems reasonable given that L1 can directly contact the P-site tRNA and shows coordination with A-site events [8, 9, 12, 71, 72]. As we know that L1 makes contacts with the P-site tRNA during hybrid state formation, loss of L1 may de-coordinate the racheting motion of the ribosome and the movement of tRNAs through the ribosome during translation. Most of the suppressors affecting the ribosome have the potential to "stiffen" the ribosome, which could help enforce order on disordered ribosomal or tRNA motion.

DbpA is one of the five DEAD-box helicases in *E. coli.* DbpA specifically recognizes helix 92 of the 23S rRNA [73, 74]. This helix is an integral component of the peptidyl transferase center of the ribosome [75]. While deletion of DbpA has no published phenotype, over-expression of ATPase-mutant DbpA results in accumulation of late phase ribosome-assembly particles [76] and a slow growth phenotype that is worse in the cold [77]. Interestingly, *dbpA* uses a GUG codon to initiate translation [78]. Given the decrease in translation from GUG start codons in the ΔL1 mutant, induction of p*dbpA* may suppress partially or wholly by correcting a reduction in translation in the absence of L1.

The dual GTPase protein EngA is an essential protein that binds at the inter-subunit face of the 50S ribosomal subunit, and is involved in 50S maturation [79]. Like

DbpA, EngA makes contacts in the peptidyl transferase center (PTC) [80]. It also fills the P and E sites and makes contact with L1 in WT ribosomes, forcing the ribosome to adopt an immature form that may represent its *in vivo* binding substrate [80]. EngA depletion results in an accumulation of 30S subunits and immature 50S subunits as well as pre-23S and pre-16S rRNAs [79, 81, 82]. A screen for *E. coli* mutants that required the otherwise dispensable ribosomal protein L9, which sits at the base of the L1 stalk, returned multiple mutants in EngA [83]. Overexpression of EngA suppresses a Δ*rrmJ* mutation, suggesting that EngA may help stabilize the PTC, reducing its flexibility [80]. It is conceivable that in the absence of L1, movement of the L1 arm is less coordinated, and that a more stereotyped, less flexible PTC helps to maintain order in the ribosomal conformational changes that are an integral part of translation. The requirement of EngA mutants for L9, which resides at the base of the L1 stalk and might also restrain the movement of the L1 arm, supports this supposition. Depletion of EngA in the presence of L1 increased the sensitivity of the ribosome to aminoglycoside antibiotics, which target A-site decoding [84]. EngA binds ppGpp as well as it binds GTP, raising the possibility of an intersection with RpoS [84].

The ribosomal protein L13 (*rplM*) is alsa near the PTC. Probing with photo-labile RNA complimentary to the 2475 loop resulted in tagging of both L1 and L13, as well as rRNA nucleotides 2470-2472, located near the PTC [85]. Other work using modified tRNAs resulted in labeling of L13 by the P-site tRNA only after cognate tRNA binding in the A-site (and presumably peptide bond formation) [86]. This

puts L13 interacting with tRNA at a stage in elongation where the tRNA may also be interacting with L1/the L1 arm as part of hybrid state formation. Other labeling experiments using a GDP analog on fusidic-acid stabilized 70S ribosomes (modeling the G-protein bound state) resulted in labeling of both L11 and L13, suggesting L13 may also contact the G-protein binding region [87]. Soung et al. also characterize L13 as part of the SRL region [88]. L13 may suppress the ΔL1 defect by aiding in appropriate passage of the tRNA into and through the E-site in the absence of L1-mediated contacts, or it may help coordinate A and E site activities if loss of L1 de-coordinates ribosome motion. Possibly, L13 associates less strongly with the ribosome in the absence of L1, and so higher cellular levels of L13 are required for normal L13 levels on the ribosome. L13 experiences significant conformational changes during amino acid starvation, and shows altered accessibility in the absence of RelA, indicating that L13 [89], like many of the L1 suppressors, may intersect with the stringent response.

The zinc regulatory protein Zur also has connections to the ribosome. Zur binds to regulatory elements upstream of certain genes, including zinc uptake genes, to repress their transcription in the presence of zinc [90, 91]. The ribosome is a major reservoir of zinc in the cell, with multiple zinc-binding ribosomal proteins [92, 93]. (Interestingly, L13 (*rplM*) is one of these [92, 94] which raises another possible explanation for its activity as a high-copy suppressor.)

A number of zinc-binding ribosomal proteins have paralogs with inactivated zinc binding regions. These paralogs are hypothesized to replace their zinc-containing related proteins on the ribosome when zinc is scarce, releasing ribosome-associated zinc for other cellular uses [95]. *E. coli* contains two zinc-containing ribosomal proteins with zinc-lacking paralogs [95-97]. These are L31 and YkgM, and L36 and YkgO, respectively [95, 98, 99]. YkgM and YkgO are encoded together in a Zur-regulated operon [98]. Overproduction of Zur could titrate zinc away from DNA-bound regulatory protein, mimicking zinc-deficient conditions and de-repressing the Zur regulon, including these alternate ribosomal proteins. L36 is important for extensive stabilization of the PTC and surrounding regions [100]. Like many of the L1 suppressors, its expression is also regulated in response to ppGpp signals [101]. L31 contacts the 5S rRNA, and spans the intersubunit space [102, 103]. It is hypothesized to restrict the racheting movement of the ribosome [102, 103], a function to which L1 could also contribute. L31 can also be cross-linked to EF-G [104]. Transcription of L36 is inhibited by DksA in the presence of ppGpp.

GlyQ is the alpha subunit of the glycl-tRNA synthetase, and the first peptide encoded in the *glyRS* operon [105]. It is the primary catalytic subunit of the synthetase, and is important for binding the glycine destined to be attached to the tRNA as well as the ATP crucial to this reaction [106, 107]. The regulation of the *glyRS* operon is unknown, but could involve feedback inhibition. Regulation of *glyRS* is attuned to growth rate by an unknown mechanism [108].

DNA Replication

Interestingly, a number of the suppressors of ΔL1 slow growth in the cold are involved in DNA replication. Of these suppressors, only *cmk* is initiated from an AUG start codon. DiaA is produced from a gene using the GUG start codon shown to be under-translated in the ΔL1 mutant, and *hda* is the only gene beginning with a CUG start codon [109], which we show to be slightly over-translated in the absence of L1.

Cmk is a CMP kinase that also has slight UMP kinase activity [110]. It produces CTP from phospholipid and RNA recycling intermediates [111]. It is not an essential gene, but deletion mutants display slow growth and significant cold sensitivity[110]. Deletion results in almost normal protein synthesis, but reduced rates of DNA replication due to slowing of replication fork progression [110]. Cellular activity is sensitive to Cmk levels, as over-expression of Cmk in wild type cells also reduces growth rates [110].

DiaA and Hda both directly interact with the DNA replication machinery. DiaA promotes assembly of replication-promoting DnaA-ATP complexes at *oriC*, ensuring timely, coordinated DNA replication [112, 113]. DiaA and the helicase DnaB bind to the same or overlapping regions on DnaA, and show competion for binding to DnaA [114]. Excess DiaA has been shown to reduce transcription in some, but not all, cases, probably by preventing DnaB binding to DnaA [113-115]. Hda interacts with

DnaA-ATP and the β-clamp to hydrolyze ATP bound to DnaA, producing inactive DnaA-ADP and preventing inappropriate re-initiation of DNA replication [116].

Cmk, DiaA, and Hda have diverse functions in DNA replication, but all are able to reduce DNA replication when supplied in excess. Although it is possible that plasmid-borne *diaA* merely increases this protein to its native level, taken together, it seems more likely that the concentration of these proteins is increased to a level capable of inhibiting DNA replication. Decreasing DNA replication rate would also decrease cell division rate. Perhaps one consequence of Δ*rplA* translation differences is to reduce translation of some protein important for cell division or survival of daughter cells, and reduction of replication rate allows the cell to adequately produce this protein.

Proteins of Unknown Relevance

The first protein of this group is DhaL. DhaL is part of the dihydroxyacetone kinase (dha) system in *E. coli* and is one of three proteins required for the PEP-dependent phosphorylation of Dha [117, 118]. DhaL is encoded in an operon with *dhaK* and *dhaM* [117, 118]. DhaK binds Dha, while DhaL binds an ADP that is transiently phosphorylated by DhaM before the phosphate is passed to Dha [117, 119, 120]. DhaL-ADP also competes with DhaK for binding to the regulatory protein DhaR, and over-expression of DhaL leads to strong activation of the *dhaKLM* operon [121]. MurI is an essential glutamate racemase [122-125]. In the absence of MurI (or compensatory mutations), peptidoglycan is not synthesized sufficiently, and cells

die by lysis. MurI is also a DNA gyrase inhibitor, and cells depleted of MurI show an elongated morphology with extended nucleoid region [122, 123]. Over-expression of MurI in a wildtype background also results in elongated cells with extended nucleoid regions [123].

YbfE is a LexA-regulated, damage-inducible protein with an unkown function [111]. YbfE has a ribbon-helix-helix predicted DNA-binding domain. YbeD structurally resembles a regulatory protein, and is found upstream of *lipB*, which is involved in lipolation of proteins, including a portion of the glycine cleavage system [126, 127]. *lipB* may be transcribed from the *ybeD* promoter, and YbeD may regulate some aspect of LipB function. YaiY is located in the cell membrane, with its C-terminus in the cytoplasm [128]. The other "Y-genes" found as suppressors are completely uncharacterized. YghS is translated from a GUG start, and so may be under-expressed in the ΔL1 mutant. YegZ may be translated from a GUA start, which may also be under-translated in the mutant, but is more likely to be translated as part of the upstream *yegR* gene, which initiates with AUG.

We tested the effect of selected suppressors of ΔL1 slow growth on the ribosome profile phenotype of the deletion mutant for cultures grown at either 18° or 37° (Fig. 3.6). Because the height of the 50S peak varies greatly compared to the 70S peak in the mutant profile, we felt it necessary to consider only profiles in which the 50S peak is below the 70S peak to be suppressed. This strict definition has the

potential to miss a great many intermediate suppressors, but we felt it necessary to prevent false positives due to 50S height variation.

We found three of the four cases tested to be suppressed. Induction of p*frsA*. which we expect to affect RpoS activity, in the mutant background in cells grown in the cold resulted in a clear and striking reduction of the 50S peak characteristic of the mutant. Expression of DbpA, which affects the ribosome, also brings the tip of the 50S peak below the tip of the 70S peak in cultures grown in the cold.

We tested the effect of induction of p*engA* and p*rplM*, both ribosome-related suppressors, on the ribosome profile of cells grown at 37°. While induction of the EngA-encoding plasmid resulted in a slightly suppressed profile, showing that ribosome profile suppression is possible at 37°, the induction of the ribosomal protein gene *rplM* did not suppress the ribosome profile defect.

DISCUSSION

We see an increase in reporter protein production from an AUG start codon in a mutant that grows extremely slowly, even at 37°. This is at odds with the previously reported decrease in protein production for isolated spontaneous L1 deletion mutations [7-9]. The disparity may be because of the difference of backgrounds between their and our strains (The originally published strains also contained antibiotic-dependent mutations, while our deletion is in a clean background, to our knowledge.) We also see almost complete abolishment of translation of the reporter

plasmid from the GUG start codon. Thus, it appears that L1 (and/or L1-interacting initiation factors) is more important for initiation from GUG than from AUG.

Our reported plasmid assays also indicate reduced specificity of the A and P sites in the Δ*rplA* mutants in some cases. While reduced P-site discrimination could lead to excess initiation, the increase in A-site errors demonstrated by the increased read-through of a UGA stop codon in the deletion mutant compared to wild type most likely leads to an accumulation of dysfunctional proteins in the ΔL1 cell. This may result in reduced availability of raw materials in the cell, and explain why many of the slow-growth suppressors also tie in to ppGpp levels and the stringent response. (Incidentally, L11, which is expected to be over-expressed in the ΔL1 mutant, is required for the stringent response in *E. coli* [129, 130]. However, over-expression of L11 has not been shown to affect stringent control [129].) Slow growth and nutrient limitations are also situations where survival is mediated by the RpoS regulon, explaining the extremely large class of RpoS-related suppressors of ΔL1 slow growth in the cold.

The ribosome-associated suppressors of ΔL1 also corroborate the reporter plasmid analysis finding of some increased errors at the A and P sites. L1, the L1 arm, and the E-site are believed to have extensive effects throughout the ribosome, and to participate in both translocation and A-site specificity [10, 11, 14, 23, 24]. The majority of the ribosome acting/binding suppressors can be expected to "stiffen" the PTC region of the ribosome, making error-prone interactions less likely and helping

to maintain the normal mechanics of the ribosome. Also, these proteins are shown to contact the region of the PTC that is able to communicate with the elongation factor binding region of the ribosome [131, 132]. In fact, rRNA bases in the vicinity of L13 have been shown to specifically affect IF2 (but not other GTPase elongation factor) function [85, 133]. It is tempting to imagine some of these proteins as helping to re-establish E-site/A-site communication when L1-mediated global conformational signals are weakened.

Interestingly, induction of the ribosome-modifying proteins obtained as slow growth suppressors of ΔL1 resulted in suppression of the ribosome profile anomaly, but induction of the ribosomal protein gene *rplM* did not. It seems that the simple presence of excess L13 does not affect the ribosome as significantly as the action of the ribosome-modifying enzymes. Possibly, ribosome-bound L13 has already made whatever contribution it can to correcting the Δ*rplA* ribosome profile defect. The strain containing p*frsA,* which I propose suppresses the slow growth of ΔL1 based on FrsA's contribution to RpoS association with the core polymerase, shows strong suppression of the ribosome profile defect, suggesting that the RpoS-related suppressors (or perhaps a unique activity of FrsA) are able to affect the ribosome itself.

The vital role of L1 in translation is only beginning to be explored. The data presented here can help us better understand the consequences of the absence of L1 and begin to construct a picture of the web of interactions in which L1 is entwined.

Acknowledgements
I would like to specifically thank Valerie Forsyth for her work carrying out the lacZ reporter plasmid assays presented in figure 3.1. I would also like to thank Dr. Richard Zielke and Billy Patterson for their work on the initial ΔrplA suppressor screen on which this work is based.

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Table 3.1: Strains and plasmids

Strain or plasmid	Relevant genotype	Source
Strain		
JM4705	WT (BW23115)	
JM6865	ΔL1	Keio
JM6375	ΔL1 with spontaneous partial suppression (designated ΔL1*)	this work
Plasmid		
pSG25	wild type *lacZ re*porter plasmid	O'Connor *et al.*, 1997
pSG431	*lacZ* with GUG initiation codon	""
pSG414	*lacZ* with UUG initiation codon	""
pSG413	*lacZ* with CUG initiation codon	""
pSG415	*lacZ* with AUC initiation codon	""
pSG416	*lacZ* with AUA initiation codon	""
pSG417	*lacZ* with AUU initiation codon	""
pSG12-6	*lacZ* with premature UAG stop codon, GUU UAG GCC	""
pSG163	*lacZ* with premature UAG stop codon, CUU UAG CUA	""
pSG627	*lacZ* with premature UAA stop codon, GAA UAA GUU	""
pSG853	*lacZ* with premature UAA stop codon, GUC UAA GUU	""
pSG34-11	*lacZ* with premature UGA stop codon, GUG, UGA GCC	""
pSG3/4	*lacZ* with premature UGA stop codon, CUU UGA CUA	""
pSG12DP	*lacZ* with -1 frameshift	""
pSGlac7	*lacZ* with +1 frameshift	""
pCA24N	Aska empty vector (EV)	Aska Collection
pCA24N*rplA*	multi-copy plasmid, (*rplA*)	""
pCA24N*cmK*	multi-copy plasmid, (*cmK*)	""
pCA24N*csiE*	multi-copy plasmid, (*csiE*)	""
pCA24N*dbpA*	multi-copy plasmid, (*dbpA*)	""
pCA24N*dhaH*	multi-copy plasmid, (*dhaH*)	""
pCA24N*diaA*	multi-copy plasmid, (*diaA*)	""
pCA24N*elbA*	multi-copy plasmid, (*elbA*)	""
pCA24N*engA*	multi-copy plasmid, (*engA*)	""
pCA24N*frsA*	multi-copy plasmid, (*frsA*)	""
pCA24N*gcvA*	multi-copy plasmid, (*gcvA*)	""
pCA24N*glyQ*	multi-copy plasmid, (*glyQ*)	""
pCA24N*greA*	multi-copy plasmid, (*greA*)	""
pCA24N*greB*	multi-copy plasmid, (*greB*)	""
pCA24N*hda*	multi-copy plasmid, (*hda*)	""
pCA24N*murI*	multi-copy plasmid, (*murI*)	""
pCA24N*nhaR*	multi-copy plasmid, (*nhaR*)	""
pCA24N*rplM*	multi-copy plasmid, (*rplM*)	""
pCA24N*sspA*	multi-copy plasmid, (*sspA*)	""
pCA24N*yaiY*	multi-copy plasmid, (*yaiY*)	""
pCA24N*ybeD*	multi-copy plasmid, (*ybeD*)	""
pCA24N*ybfE*	multi-copy plasmid, (*ybfE*)	""
pCA24N*yegZ*	multi-copy plasmid, (*yegZ*)	""
pCA24N*yghS*	multi-copy plasmid, (*yghS*)	""
pCA24N*yghT*	multi-copy plasmid, (*yghT*)	""
pCA24N*yhjO*	multi-copy plasmid, (*yhjO*)	""
pCA24N*yidR*	multi-copy plasmid, (*yidR*)	""
pCA24N*yjiD*	multi-copy plasmid, (*yjiD*)	""
pCA24N*zur*	multi-copy plasmid, (*zur*)	""
pCA24N*rpoS*	multi-copy plasmid, (*rpoS*)	""

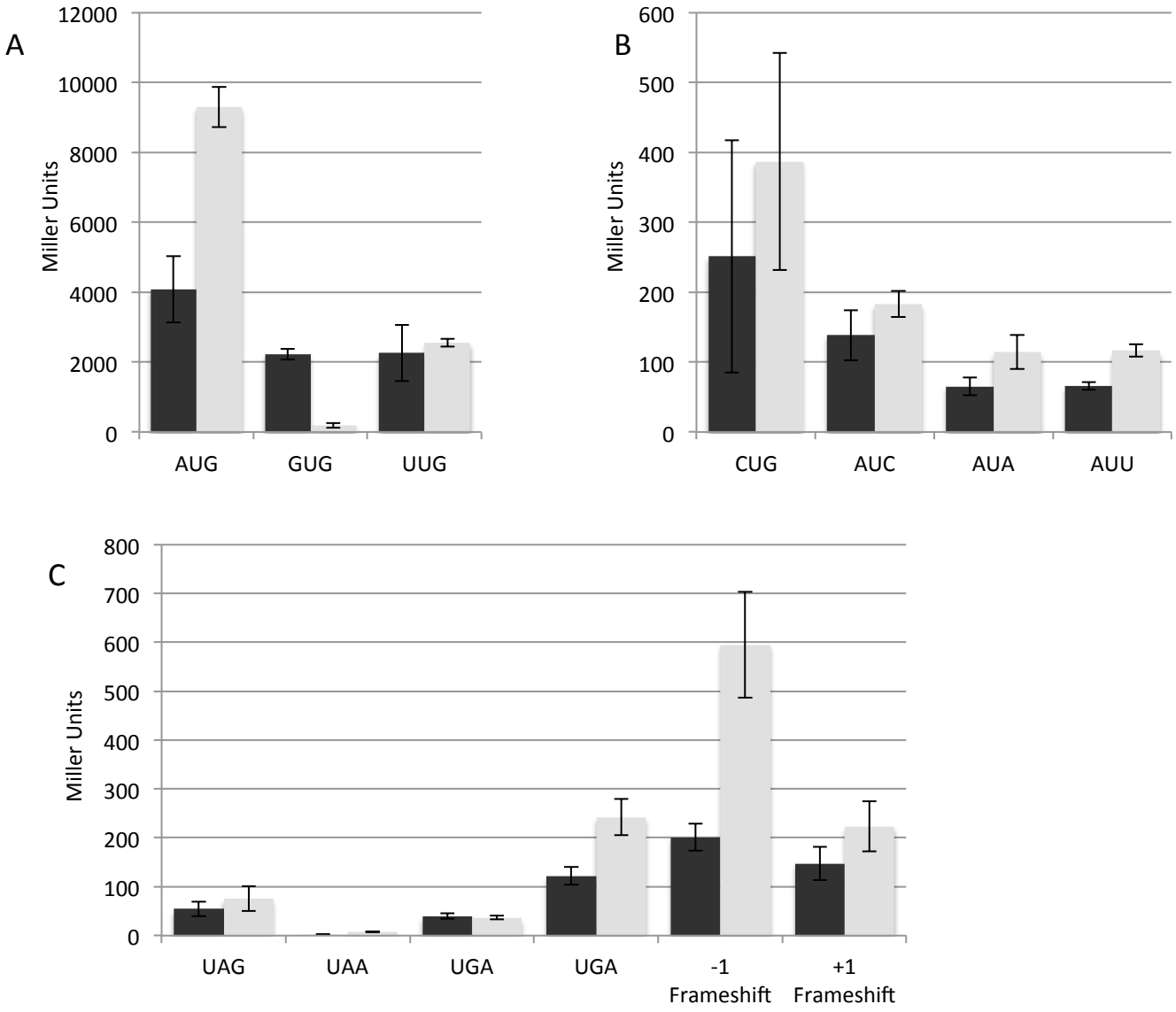


Figure 3.1: Effects of loss of ribosomal protein L1 on P-site and A-site fidelity. Translation of *lacZ* reporter plasmids was measured in WT and ΔL1 strains and reported in Miller units. . Initiation from start (A) and non-start (B) codons as well as translation through stop codons and +1/-1 reading frame shifts (C) are shown for Δ*rplA* cells ( ) and wild type cells ( ). Data represent the average of at least three replicates and error is shown in standard deviation.

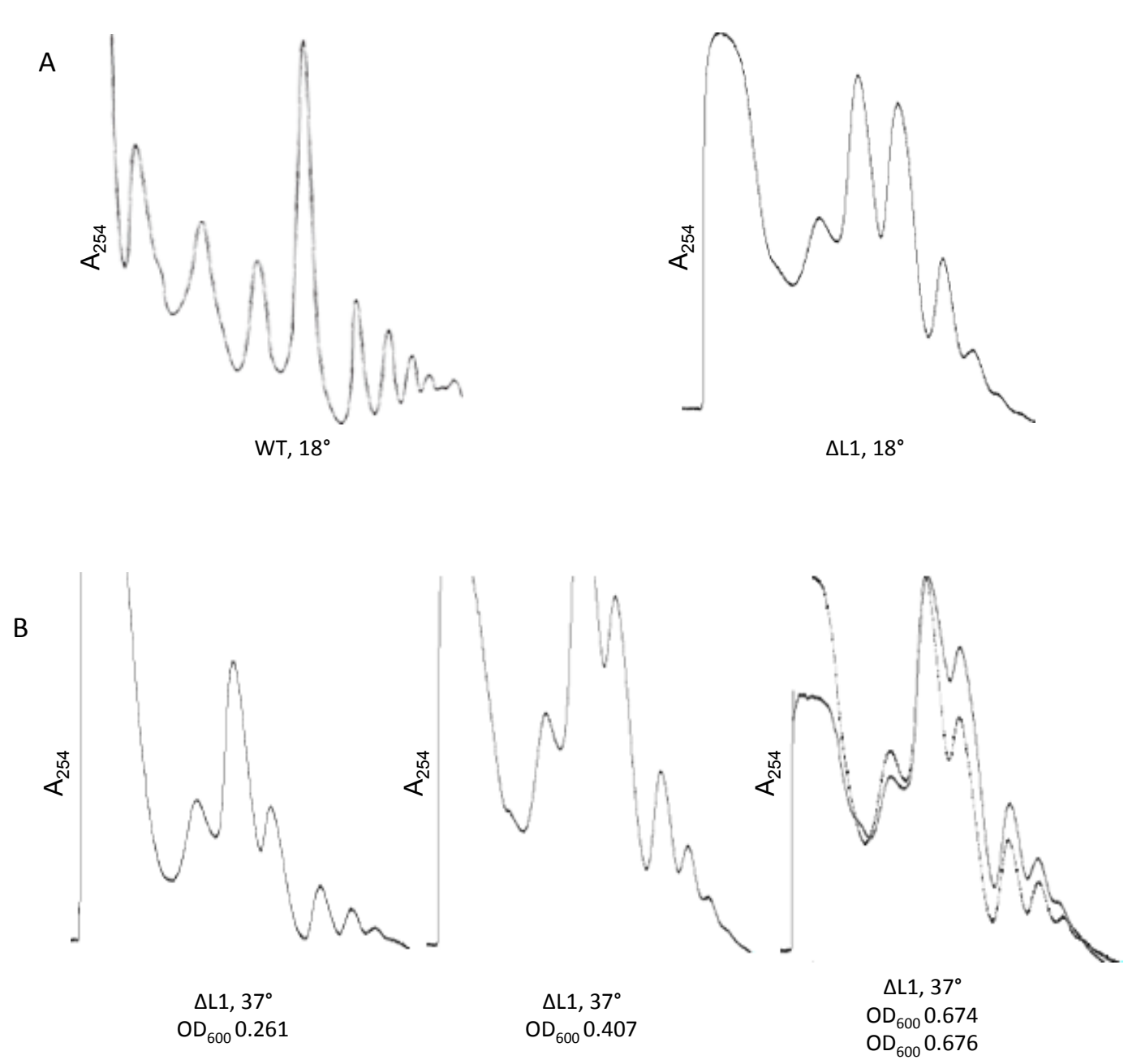
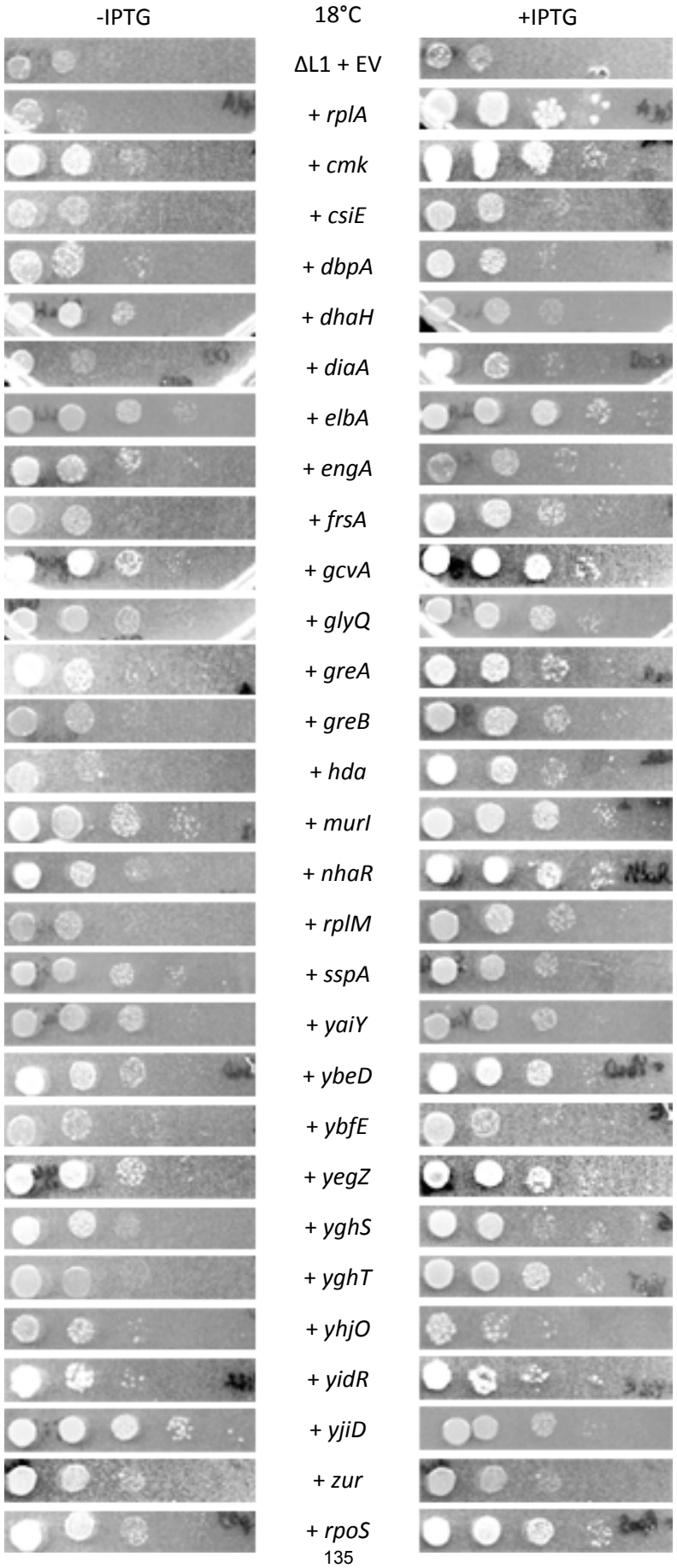


Figure 3.2: Loss of the L1 protein results in an accumulation of 50S ribosomal subunits. Clarified cell extracts of wild type and mutant strains grown at (A) 18° or (B) 37° were separated on a sucrose density gradient and pumped past a detector to obtain an A_{254} absorbance profile. In (B) replicates from multiple cultures harvested at a range of OD_{600} show variability of mutant profile.

Figure 3.3: High copy suppressors of ΔL1 cold sensitivity. A library screen was carried out to determine high-copy suppressors of the cold-sensitive slow-growth of a ΔL1 strain. Twenty-nine unique candidate suppressors were confirmed by independent transformation and growth assay by spotting dilutions of overnight cultures and outgrowth at 18°.


-IPTG
18°C
+IPTG
ΔL1 + EV
+ rplA
+ cmk
+ csiE
+ dbpA
+ dhaH
+ diaA
+ elbA
+ engA
+ frsA
+ gcvA
+ glyQ
+ greA
+ greB
+ hda
+ murI
+ nhaR
+ rplM
+ sspA
+ yaiY
+ ybeD
+ ybfE
+ yegZ
+ yghS
+ yghT
+ yhjO
+ yidR
+ yjiD
+ zur
+ rpoS

Functional Classes of ΔL1 Slow Growth in the Cold High-Copy Suppressing Proteins

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RpoS-Related Suppressors

BcsA (YhjO) CsiE FrsA GcvA GreA GreB IraD (YjiD) IraM (ElbA)
NhaR* SspA RpoS

Ribosome-Related Suppressors

DbpA EngA GlyQ RplM Zur

DNA Replication-Related Suppressors

CMK **DiaA** Hda

Other

DhaL MurI YaiY YbeD YbfE YegZ• **YghS** YghT YidR

Proteins in **BOLD** are translated from a GUG start codon
**nhaR* lacks an obvious promoter and may be translated by readthrough from *nhaA*, which uses a GUG start
•*yegZ* may use a GUA start codon, which could also be under-translated in the mutant. Alternately, *yegZ* may be translated as part of upstream *yegR* (AUG start).

Table 3.2: Functional classes of ΔL1 slow growth in the cold high-copy suppressing proteins

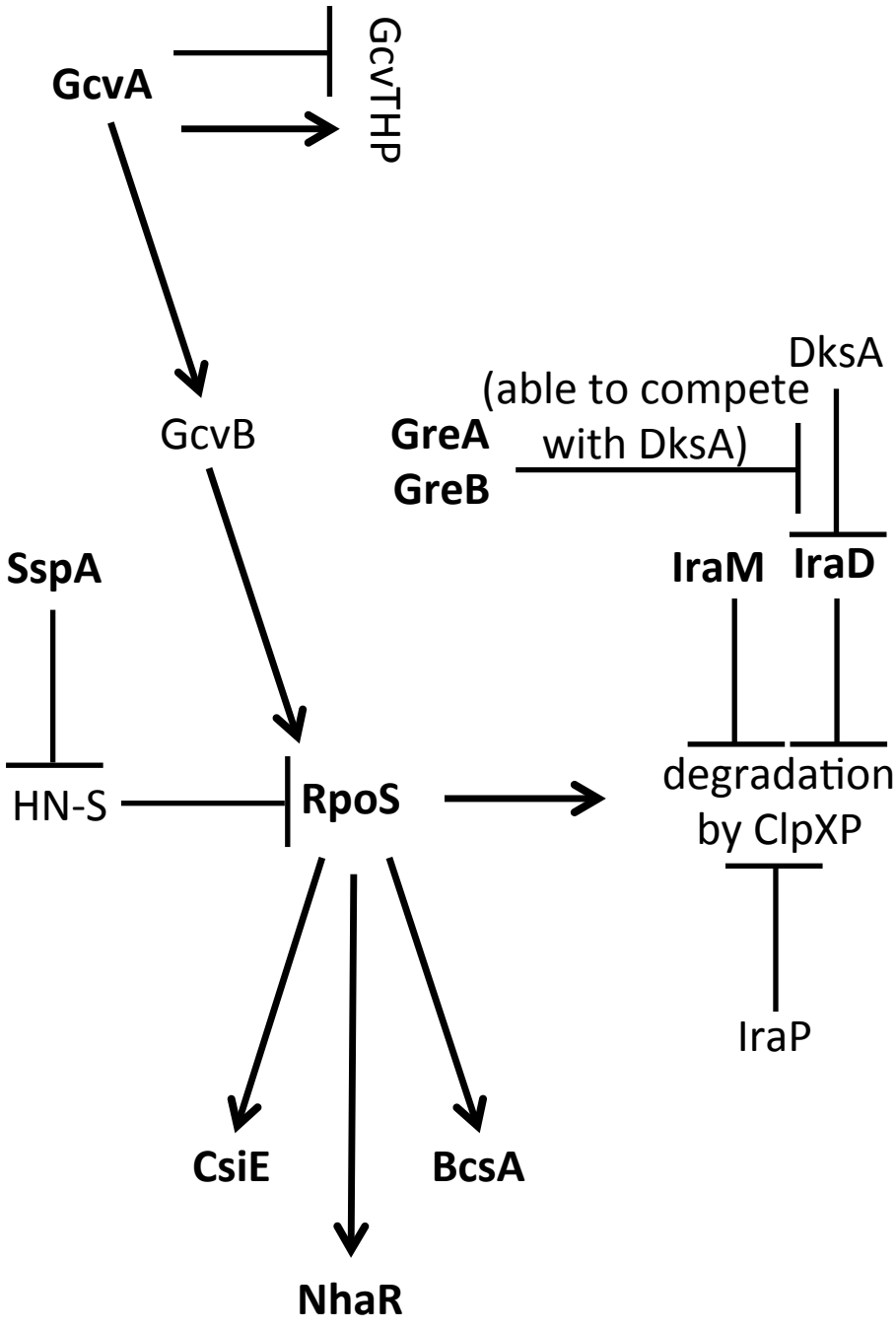

Figure 3.4: Relationship of RpoS-related high-copy suppressing proteins of ΔL1 slow growth in the cold. Confirmed suppressors are indicated in **BOLD**. Over-expression of FrsA (not shown in) may result in freeing of otherwise sequestered σ^{70} anti-adapter, promoting σ^{S} competition for the core polymerase.

Adapted from Petrov et al. 2013

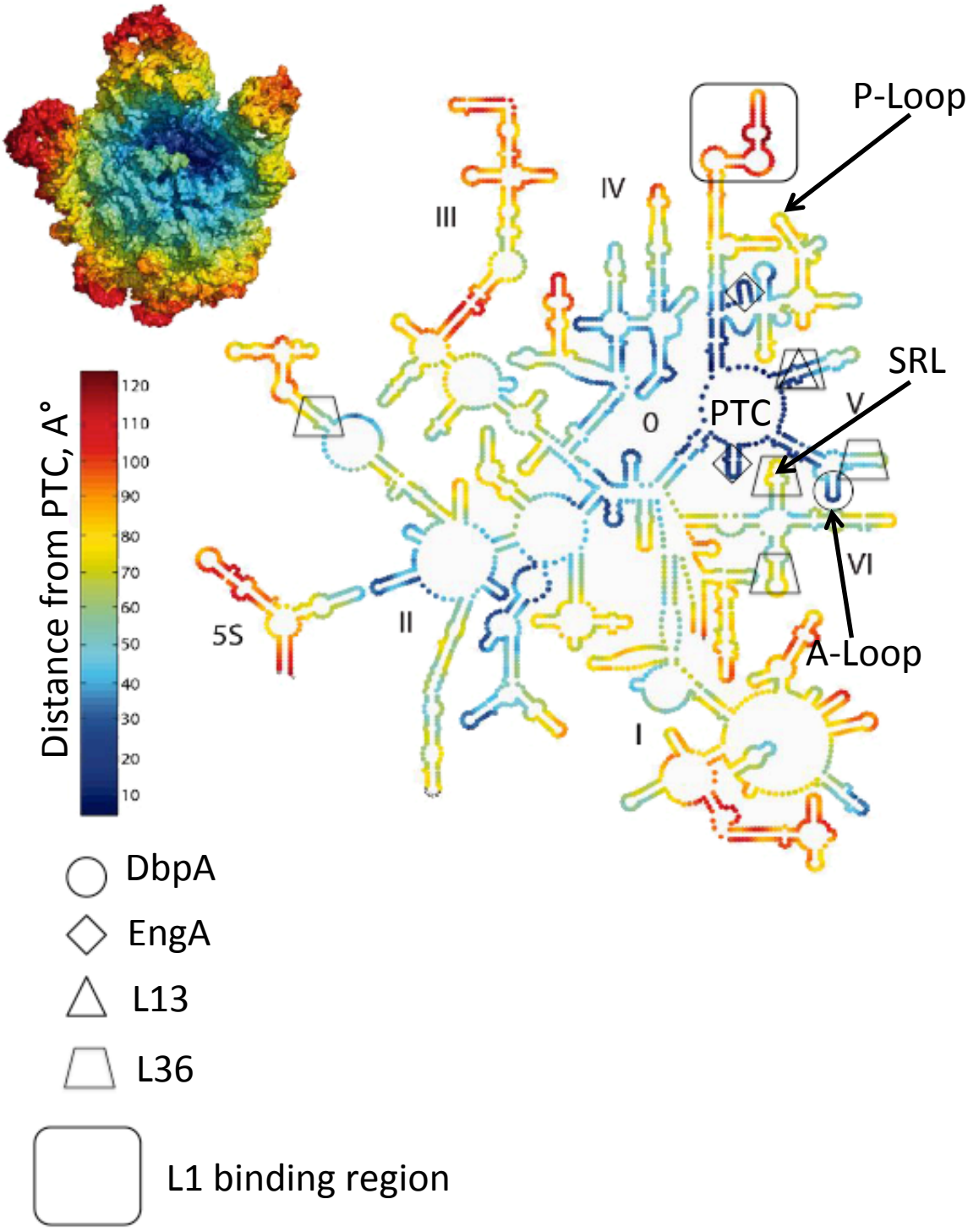

Figure 3.5: The ribosome-binding/affecting sites of ribosome-related suppressing proteins clusters around the PTC. Experimentally-derived binding, acting, and protecting locations of ribosome-related high copy suppressing proteins of ΔL1 slow growth in the cold are shown on a HEAT style map of the ribosome, where dark blue represents the PTC and red represents the least PTC-associated portions of the ribosome. The L1 binding site is also marked. L31 (not on figure), the other protein possibly affected by Zur over-expression, makes contact with the 5S rRNA, but is primarily associated via protein-protein interactions with both the large and small subunits. Roman numerals indicate rRNA domains. PTC: Peptidyl Transferase Center, SRL: Sarcin-Ricin Loop

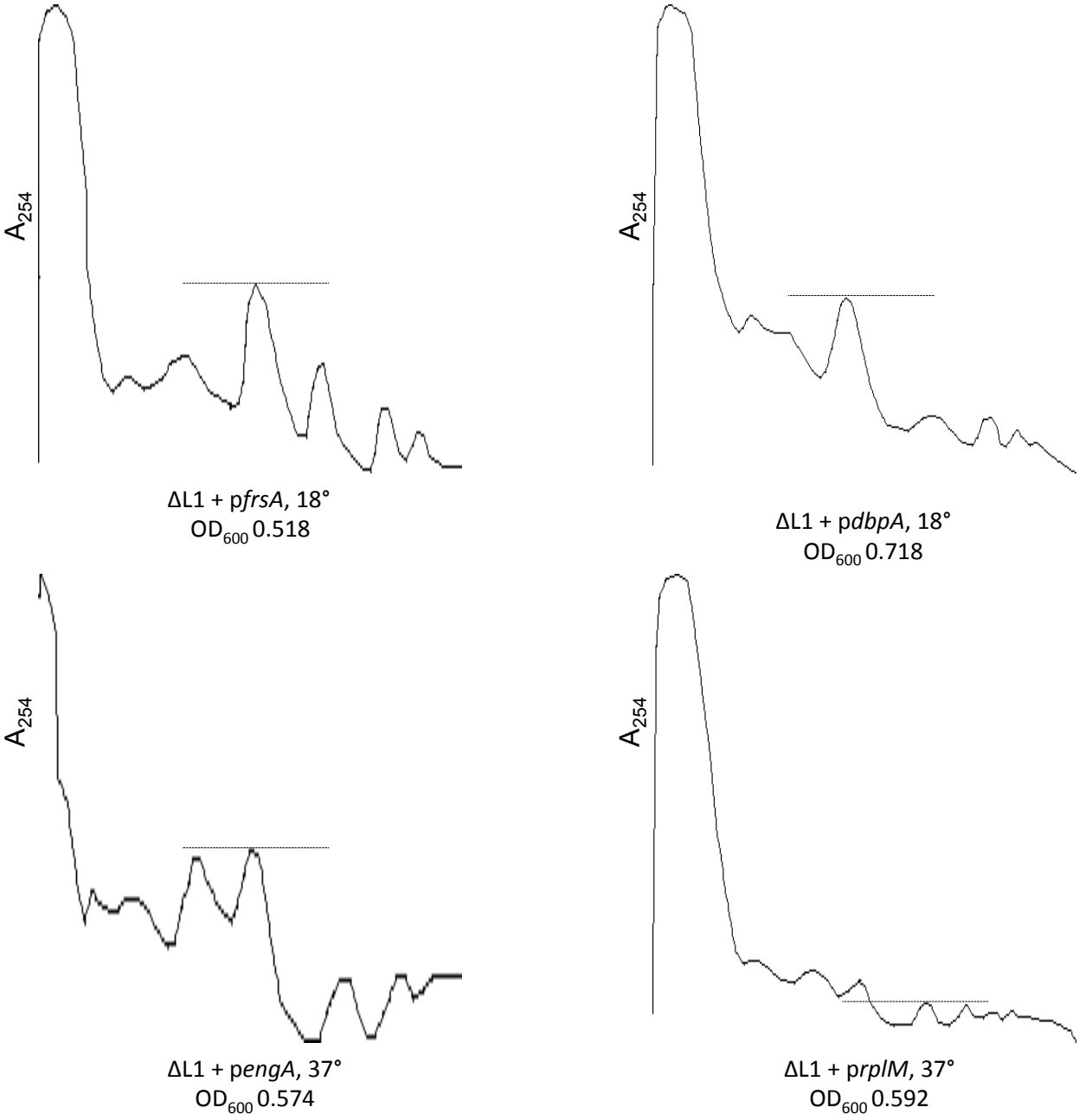

Figure 3.6: Not all the high-copy suppressors of ΔL1 slow growth also suppress the ΔL1 ribosome profile defect. The ribosome profiles of ΔL1 cultures with high-copy suppressor plasmids grown in the presence of 25μM IPTG at the temperatures listed are shown. Cultures were harvested at the OD_{600} stated, then lysed, clarified, and subjected to sucrose density gradient ultracentrifugation before their OD_{254} profiles were recorded. Because of the variability in the height of the 50S peak compared to the 70S peak in ΔL1 ribosome profiles, only profiles where the 50S peak was lower than the 70S peak were considered suppressed. Horizontal lines show the tip of the 70S peak.

Chapter 4

Future Directions

Based on the similarity of Uup to EttA, and on the data available for these two proteins, I have proposed that Uup sits in the E-site of a ribosome containing an initiator tRNA ($tRNA^{fMet}$) and an encoded P-site elongator tRNA and promotes formation of the first peptide bond. While my data is consistent with a such a function, and the conservation of residues important for recognizing $tRNA^{fMet}$ [1] between Uup and EttA makes an interaction with the initiator tRNA seem likely, there is still much work to do to support (or disprove) this model. In addition, the lack of a striking growth defect in Δ*uup* mutants means it is highly possible that Uup only exerts its influence on a subset of ribosomes. Elucidating the unique features of these ribosomes would go far towards fleshing out my model of Uup function and elucidating its role in ribosome regulation. Finally, clarification of the relationship between Uup and BipA would go far in elucidating the function of the most enigmatic member of the translational GTPase family, as well as provide a more nuanced understanding of cellular control of the central cellular process of protein production.

The first step towards supporting my model of Uup as an E-site factor promoting formation of the first peptide bond is to examine whether Uup actually sits in the E-site of the ribosome. My proposed ribosome binding of Uup is based on the recently-published structure of EttA on the ribosome [1]. Cryo-EM or X-ray crystallography studies establishing the general ribosome binding site of Uup is vital in determining the validity of my model. Further, It must be determined whether the P-site tRNA interacting motif of Uup makes the expected contacts with residues specific for the initiator tRNA. If these contacts are made, then the specificity of the Uup-initiator tRNA interaction should be tested. This could be done by incubating a combination of ribosomes programmed with either a $tRNA^{fMet}$ or an elongator tRNA in the P-site (and an elongator tRNA in the A-site) with a limited amount of Uup followed by pull-down of the Uup-ribosome complexes and analysis of the tRNA content of the Uup-bound ribosomes [2]. If Uup binds specifically to ribosomes containing an initiator tRNA in the P-site, then isolated Uup-bound ribosomes should contain a higher proportion of initiator vs. elongator tRNA compared to the original programmed ribosome mixture. Such an approach could be complicated by our observation that Uup seems to "fall off" ribosomes relatively easily during rough treatment such as sucrose gradient centrifugation. Use of ATPase-null Uup mutants or reversible cross-linking may be able to overcome this potential difficulty. Another way to test whether Uup preferentially interacts with ribosomes bearing $tRNA^{fMet}$ is to compare the effect of Uup on *in vitro* translation assays [2]using ribosomes pre-programmed with either initiator or elongator tRNA in the P-site.

I expect Uup to make contact with the ribosomal protein L1 (encoded by *rplA*) in a manner similar to EttA. In experiments testing the effect of high-copy mutant Uup in a Δ*rplA* background, the induction of p*uup2* was usually dominant negative, as seen in wild type and Δ*bipA* backgrounds. However, in the Δ*rplA* background, mutants that were insensitive to Uup2 regularly appeared. I found that this was due to changes in the genetic background of the strain, not to mutations of the plasmid (data not shown). In addition to L1, EttA also makes contact with other large and small subunit proteins and with the rRNA. It would be useful to sequence the genomes of the Uup2-insensitive Δ*rplA* mutants and determine whether they harbored mutations in proteins/RNA expected to interact with Uup or in other areas that could provide additional insight into the function of Uup. This could help confirm the proposed E-site binding site of Uup on the ribosome, or suggest other interacting partners for Uup.

The next tenet of my model that requires direct testing is the proposal that Uup promotes formation of the first peptide bond, particularly when ATP is abundant. This can be tested in a limited *in vitro* translation assay as done for EttA [2]. It will be important to compare the effect of Uup at high and low ATP:ADP ratios in order to clarify under which conditions Uup has most effect. Repeating these studies with increasing levels of EttA would allow us to confirm or deny our expectation that high levels of EttA can interfere with Uup function, most likely by out-competing Uup from the ribosome. As high-copy Uup suppresses several cold-sensitive phenotpyes of Δ*bipA* mutants, it would also be instructive to compare the effect of

Uup between *in vitro* translation assays using ribosomes isolated from cultures grown at 37°C or 18°C.

Because there is no obvious growth defect in the Δ*uup* mutant, it seems unlikely that Uup is required during every round of initiation. It is possible that Uup is required only for efficient translation of certain messages, under certain environmental conditions, or for initiation complexes in which the components are slightly mis-aligned. If Uup is important for the translation of specific messages, then mRNA for DNA-binding proteins that share a transposon excision (*tex*) defect with Uup [3] are logical potential targets. Western blotting of wild type and Δ*uup* cell cultures using antibodies against Ssb, PolA, and TopA (at both 37°C and 18°C) followed by quantification of the bands under each condition may show large differences in protein expression, if Uup is important for their translation. A more direct test would be to compare *in vitro* translation of mRNA for each of these proteins in the presence and absence of Uup to see whether translation was more efficient for any of these messages in the presence of Uup. *In vivo*, *lacZ* reporter gene expression from each *tex* gene promoter region could be quantified and compared in wild type and Δ*uup* strains [4]. Finally, for genes on this list for which viable mutants exist whose severity of phenotype varies with expression level, in vivo tests comparing the phenotype with and without high-copy Uup (similar to my work with *topA* mutant presented earlier, [Chapter 2, this work]) could be used to test the biological relevance of differences found by other methods. To cast a wider net in search of specific mRNA targets of Uup, proteomics could be used to search for proteins that

are less abundant in Δ*uup* strains compared to wild type cells. Ribosome profiling (as used to determine targets of the E-site factor EF-P [5]) could be used to find mRNAs that are translated less efficiently in Δ*uup* strains compared to wild type cells. If no candidates are found by any of these methods, then a role for Uup under certain environmental conditions (possibly cold temperatures, where the kinetic energy of the ribosomal components is less) or in cases of not-quite-right initiation complex assembly is more likely. Since the observable growth consequences are minimal in a Δ*uup* mutant, Uup is unlikely to act in every round of translation even under the specific circumstances tested to date.

Another critical area for exploration is the relationship between Uup and the translational GTPase BipA. High-copy *uup* suppresses many of the defects seen in Δ*bipA* strains at 18°C. A paper published after writing of this document revealed the cryo-EM structure structure of BipA on the ribosome [6]. The unique C-terminal domain of BipA reaches into the A-site of the ribosome and makes contact with helix 92 of the ribosomal peptidyl transferase center. Helix 92 binds and positions the amino-acyl acceptor region of the A-site tRNA during protein synthesis. The BipA-bound ribosome takes on a partially racheted conformation, in which the 30S subunit is rotated in the same direction and to a comparable degree as in an EF-G bound pre-translocation complex. However, the 30S head swivel necessary to allow translocation of the P-site ribosome to the E-site is much smaller in the BipA-bound ribosome than in the EF-G bound pre-translocation ribosome. In contrast, the 30S subunit rotates in the opposite direction in the LepA-bound ribosome [7]. This

similarity in ribosome rearrangement in the EF-G and BipA-bound states provides a structural explanation for our finding that high-copy *fusA* (encoding EF-G) partially suppresses a Δ*bipA* phenotype.

BipA is shown bound to a ribosome containing tRNA in the A, P, and E sites. The A-site tRNA is in an essentially classical position in the BipA-bound ribosome, and the E-site tRNA shows only a small movement towards the L1 side of the ribosome. The P-site tRNA, however, shows a significant displacement of its 50S interacting end towards the E-site of the ribosome in the BipA-bound structure compared to its classical position. The L1 arm is seen in an open position, a pre-requisite for exit of the E-site tRNA from the ribosome. The presence of BipA on ribosomes containing an A-site tRNA indicates that Uup and BipA can bind to ribosomes with similar tRNA occupancy.

In order for Uup to bind to the ribosome, the E-site must be empty. The E-site must also be empty for the L1 arm to play its role in EF-G mediated translocation. To date, there is no protein shown to mediate exit of the E-site tRNA from the ribosome. The tRNA is modeled as leaving due to diffusion mediated by kinetic energy of the E-site tRNA, possibly in coordination with allosteric signals from the A-site of the ribosome [8]. In the cold, the kinetic energy of the E-site tRNA may be too low to consistently result in tRNA exit from the ribosome. The changes in E and P-site tRNA position in the BipA-bound ribosome could be interpreted as promoting exit of the E-site tRNA. Since the data so far indicates that absence of BipA is much

more harmful in the cold than at 37°C or higher temperatures [9, 10], perhaps BipA promotes emptying of the E-site in the cold. In this model then, Uup would promote a single step initiating elongation, while BipA would act to keep the elongation cycle moving forward. Slowing both processes, as in the Δ*bipA*Δ*uup* double mutant, would result in a cumulative decrease in protein production that could explain the dramatic growth defect of the double mutant in the cold.

Our (unpublished) data indicates that BipA is particularly important for the translation of certain mRNAs. Creation of an *in vitro* translation assay using these mRNAs and reflecting the importance of BipA in the cold would allow us to test whether Uup can also contribute to translation under these specific circumstances.

The recent advances in knowledge of both A-site and E-site translation factors represents exciting progress in our understanding of translation. The probability that some or all of these factors act under specific circumstances or on specific mRNAs highlights the complexity of translation, and leaves as many questions as answers. It seems that we are poised to probe a new layer of complexity in ribosome function. Uup, EttA, and BipA may lead the way.

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