The Pennsylvania State University

Eberly College of Science

Department of Biochemistry and Molecular Biology

RIBOSOME RESCUE IS ESSENTIAL IN BACTERIA AND HUMAN CELLS

A Dissertation in

Biochemistry, Microbiology, and Molecular Biology

by

Heather A. Feaga

Submitted in Partial Fulfillment

of the Requirements

for the Degree of

Doctor of Philosophy

August 2016

The dissertation of Heather Feaga was reviewed and approved* by the following:

Kenneth Keiler
Professor of Biochemistry and Molecular Biology
Dissertation Advisor
Chair of Committee

Richard Frisque
Professor of Molecular Virology
Associate Department Head for Equity and Diversity

Katsuhiko Murakami
Professor of Biochemistry and Molecular Biology

B. Tracy Nixon
Professor of Biochemistry and Molecular Biology

K. Sandeep Prabhu
Professor of Immunology and Molecular Toxicology

Scott Selleck
Professor of Biochemistry and Molecular Biology
Head of the Department of Biochemistry and Molecular Biology

*Signatures are on file in the Graduate School

ABSTRACT

All life on earth relies on the ability to translate the genetic code into protein. DNA is transcribed first into mRNA, which is then decoded by the ribosome to produce all of the proteins in the cell. The ribosome is a large, multi-subunit entropy trap that catalyzes the assembly of each protein based on a series of codons provided by the mRNA. This reaction has been occurring for billions of years and is essential in every organism. As a result, the process is highly efficient, and extensive quality control mechanisms have evolved to maintain the fidelity of each step of protein synthesis, from initiation to termination. Ribosomes are extremely stable and the small and large subunits of the ribosome make extensive contacts with each other and with a long stretch of the mRNA. Additionally, the peptidyl transfer center maintains a water-tight seal to protect the peptidyl-tRNA from hydrolysis. The translation complex is therefore extremely stable, and must be actively disassembled after each round of translation. At the end of each protein coding reading frame, a stop codon signals the end of the peptide. Release factors make specific contacts with the stop codon and position a water molecule in the peptidyl transfer center to hydrolyze the peptidyl tRNA. Recycling factors can then disassemble the ribosome for a new round of translation. If no stop codon is present, the ribosome can not elongate or terminate the peptide. Instead, the ribosome is sequestered in a nonstop translation complex with the mRNA still bound and a peptidyl-tRNA remaining in the P site. Nonstop translation complexes arise so frequently that some estimates predict that each ribosome is bound up in a nonstop translation complex 5 times per cell division cycle. The findings in this dissertation will show that resolving nonstop translation complexes is essential in bacteria and human cells.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

abbreviation	expansion
ArfB	alternative rescue factor B
ICT1	immature colon carcinoma transcript-1
PYE	peptone yeast extract
KKL-35	Ken Keiler Lab molecule 35
Tn-seq	transposon mutagenesis followed by deep sequencing of insertion sites
RF-1	release factor 1
RF-2	release factor 2
eRF1	eukaryotic release factor 1
eRF3	eukaryotic release factor 3
OD	optical density
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
HTS	High-throughput screening
DHFR	dihydrofolate reductase
cat	chloramphenicol acetyltransferase
PATRIC	pathosystems resource integration center
siRNA	silencing RNA
siICT1	silencing RNA targeting ICT1 transcript
siNT	non targeting RNA

ACKNOWLEDGEMENTS

It has been a privilege to work with my adviser, Ken Keiler, who has held me to a high standard while giving me all the tools I need to live up to that standard. He has taught me so much about how to ask good scientific questions, solve problems, and communicate effectively. His lab provided me with the best possible environment to do the work I love so much, and I couldn't have asked for better training. I am so proud of all the work the Keiler lab has done. Ken not only discovered *trans*-translation during his graduate work, but then found drugs to target this pathway that are effective against many pathogens. These drugs may one day be used to treat bacterial infections in humans, and we need these new drugs desperately. I have always felt deeply fortunate to be connected to this research.

I have been aided by many very talented collaborators, most notably Mike Quickel of the Hankey-Giblin lab at Penn State who performed the human cell experiments reported in chapter 3. Also, Patrick Viollier of the University of Geneva who constructed the *Caulobacter crescentus* transposon library used for the Tn-seq experiment described in chapter 2. I am also grateful to So Woon (Sophie) Kim, an undergraduate in the Keiler Lab who helped with some early attempts to identify the alternative rescue system in *Bacillus subtilis*.

I am grateful to my thesis committee, Dick Frisque, Katsu Murakami, Tracy Nixon, and Sandeep Prabhu, for their willingness to give me so many hours of one on one time to offer both advice and support during my development of this project. I am grateful to Sarah Ades for providing so many strains and helpful advice.

I have been fortunate to enjoy the support of many labmates who have grown into close friends and have taught me so much. Sarah Muse, Sara Hester, and Laura Goodfield, taught me some of my very first skills as a microbiologist and guided me through some of the initial challenges of my graduate career. Alexia Karanikas and Jihye Park endlessly inspired me with

their perseverance and kindness. Liron Bendor and David Place kept me thinking broadly and creatively. Shaima El-Mowafi remains a true friend and an example of real courage. John Alumasa has helped so much with his endless postdoc wisdom. Amber Miller has been a great source of support.

I am grateful to my dad Howie Feaga, a dairy farmer, and my mom Wendy Feaga, a pastor and veterinarian. Thank you for instilling in me curiosity and self-sufficiency, and thank you for setting an example of how to problem solve and keep moving forward in spite of adversity. I am grateful to many other friends and family including Annie Feaga, Laura Feaga, Kristin Kearney, Emersyn Kearney, Pam Jadwick, Leland Powell, Linda Baum, and my first mentor in research, Veronika Szalai, with whom I am still in touch and who has given me continued support and encouragement. Finally, I want to acknowledge my husband, Brooks Crickard, for his incredible support and partnership. He contributed tremendously to all of this work with so much helpful discussion and endless interest. He has at all times had complete confidence in me. Thank you all so much!

Chapter 1

INTRODUCTION: Ribosome rescue is a matter of life and death

Adapted from:

Keiler KC, Feaga HA. 2014. Resolving nonstop translation complexes is a matter of life or death. *Journal of Bacteriology*. 196:1–9.

Abstract

Problems during gene expression can result in a ribosome that has translated to the 3' end of an mRNA without terminating at a stop codon, forming a nonstop translation complex. The nonstop translation complex contains a ribosome with the mRNA and peptidyl-tRNA engaged, but because there is no codon in the A site the ribosome cannot elongate or terminate the nascent chain. Recent work has illuminated the importance of resolving these nonstop complexes in bacteria. tmRNA-SmpB specifically recognizes and resolves nonstop translation complexes in a reaction known as *trans*-translation. *trans*-Translation releases the ribosome and promotes degradation of the incomplete nascent polypeptide and problematic mRNA. tmRNA and SmpB have been found in all bacteria, and are essential in some species. However, other bacteria can live without *trans*-translation because they have one of the alternative release factors, ArfA or ArfB. ArfA recruits RF2 to nonstop translation complexes to promote hydrolysis of the peptidyl-tRNAs. ArfB recognizes nonstop translation complexes in a manner similar to tmRNA-SmpB, and directly hydrolyzes the peptidyl-tRNAs to release the stalled ribosomes. Genetic studies indicate that most or all species require at least one mechanism to resolve nonstop translation complexes. Consistent with such a requirement, small molecules that inhibit resolution of nonstop translation complexes have broad-spectrum antibacterial activity. Eukaryotic cells use paralogs of eRF1 and eRF3, called DOM34 and Hbs1 respectively, to disassemble ribosomes from nonstop mRNA in the cytoplasm. All eukaryotic cells encode an ArfB homolog named ICT1. In human cells, ICT1 is imported into mitochondria. ICT1 is essential in human cells, indicating that nonstop translation complexes must be resolved in mitochondria to maintain viability. Altogether, these results suggest that resolving nonstop translation complexes is a matter of life or death for both bacteria and eukaryotic cells.

Resolution of nonstop translation complexes in bacteria

Bacteria perform transcription and translation in the same cellular compartment because they do not have nuclei. One advantage to this arrangement is that bacteria can rapidly respond to environmental challenges by producing new proteins. The time between transcription of a gene and the availability of the corresponding protein is minimized because the mRNA does not have to be processed or exported, and translation of an mRNA can initiate before transcription is complete. However, using a single compartment for transcription and translation has serious consequences for protein quality control because there are limited opportunities for mRNA proof-reading. Mechanisms used by eukaryotes to ensure the mRNA is intact are generally absent in bacteria. For example, in eukaryotes 3' polyadenylation is used as a signal that the mRNA transcript is complete. This signal is read at several steps, including nuclear export and translation initiation, which requires interaction between poly(A)-binding proteins and translation initiation factors (1,2). In contrast, the bacterial ribosome does not require any information from the 3' end of the mRNA to initiate translation, so there is no assurance that the mRNA is complete or intact (3). As a consequence, bacterial ribosomes frequently translate mRNAs that do not have a stop codon ("nonstop" mRNAs). When a ribosome reaches the 3' end of a nonstop mRNA, it is trapped in a nonstop translation complex. In this complex, the mRNA and peptidyl-tRNA in the P site prevent dissociation of the ribosome, but the complex cannot elongate or terminate because there is no codon in the A site. A nonstop complex can also be formed when a ribosome stalls during translation and the mRNA is cleaved in the A site (4-6). Estimates from *E. coli* suggest that 2-4% of translation reactions end in a nonstop translation complex (7). At this rate, an

average ribosome will be involved in ~5 nonstop translation complexes per cell division. Clearly, the protein synthesis capacity of the cell would be severely compromised if these complexes could not be quickly resolved. To cope with the prevalence of nonstop translation complexes, bacteria have a remarkable mechanism known as *trans*-translation, which can release the ribosome and target the nonstop mRNA and nascent polypeptide for rapid degradation.

Resolution of nonstop translation complexes by tmRNA-SmpB

trans-Translation is performed by a ribonucleoprotein complex consisting of tmRNA, a specialized RNA molecule, and SmpB, a small protein. tmRNA has elements of both a tRNA and an mRNA. The 5' and 3' ends of tmRNA form a structure resembling the acceptor arm and TΨC arm of alanyl-tRNA (8,9). The remainder of tmRNA includes several pseudoknots and a specialized reading frame that is decoded during *trans*-translation (8,10-12). SmpB binds tmRNA and completes the tRNA-like structure by mimicking the anticodon stem (13-15). The acceptor arm of tmRNA is charged with alanine by alanyl-tRNA synthetase and bound by EF-Tu in the same manner as $tRNA^{Ala}$ (8,16,17). During *trans*-translation, tmRNA-SmpB specifically recognizes a nonstop translation complex and is accommodated in the ribosomal A site (Figure 1-1) (18-21).

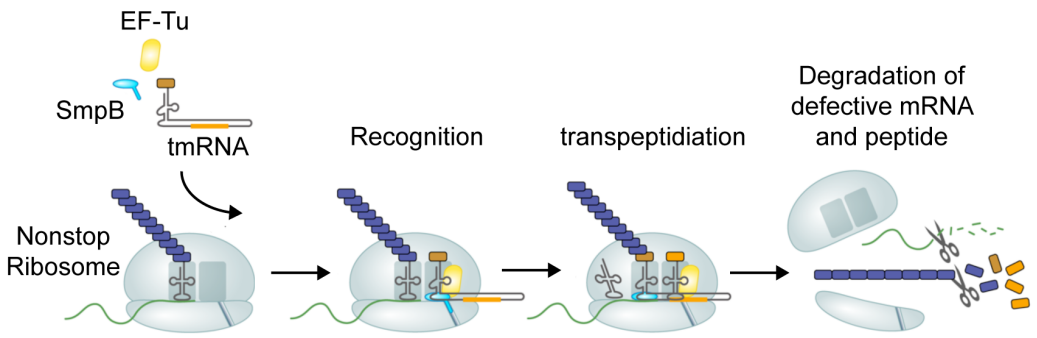

Figure **1-1:** Schematic of *trans*-translation. During *trans*-translation, tmRNA-SmpB recognizes nonstop translation complexes by binding in the empty mRNA channel and uses a reading frame within tmRNA to mediate the release of the ribosome and target the nascent polypeptide for proteolysis. The problematic mRNA is also degraded.

The nascent polypeptide is transferred to the alanine charged to tmRNA, and SmpB-tmRNA is translocated to the P site. During translocation, a large swivel of the 30S head of the ribosome allows the reading frame of tmRNA to enter the mRNA channel (22). The first codon of the tmRNA reading frame is aligned in the A site, and translation resumes using the tmRNA reading frame as a message. Correct alignment of tmRNA in the mRNA channel requires sequence-specific contacts with SmpB (23). Translation of the tmRNA reading frame terminates at a stop codon, releasing the ribosome and a protein that includes the tmRNA-encoded peptide tag at the C terminus (24). The peptide tag is recognized by multiple proteases in the cell, ensuring that the protein is rapidly degraded (24-28). The nonstop mRNA is also targeted for degradation during *trans*-translation (29-31). Thus, the overall effect of the reaction is to remove the problematic mRNA and the incomplete protein, and to release the ribosome.

A crystal structure from Neubauer, et al., captures an early step of *trans*-translation and shows how tmRNA-SmpB recognizes nonstop translation complexes (32) (Figure 1-2). In the structure, the tRNA-like domain of tmRNA, bound with SmpB and EF-Tu, is trapped in the A site of a nonstop translation complex during accommodation using the drug kirromycin. Overall,

the structure resembles an elongation complex with tmRNA-SmpB in place of the acylated tRNA. The acceptor arm of tmRNA is in the same orientation as the acceptor arm of the acylated tRNA, and SmpB takes the place of the anticodon stem. However, SmpB also makes contacts in the decoding center and empty mRNA channel that appear to mimic the missing mRNA. The 16S rRNA residues A1492, A1493, and G530, which interact with the mRNA in an elongation complex, directly contact SmpB in the nonstop complex. In addition, the C terminus of SmpB forms a helix that extends into the empty mRNA channel between the decoding center and the leading edge of the ribosome. Chemical footprinting and mutational studies support the presence of these interactions during *trans*-translation (33,34). This crystal structure suggests that tmRNA-SmpB could not be accommodated in elongating ribosomes because the mRNA would obstruct SmpB binding. Consistent with this model, competition experiments show that tmRNA-SmpB does not interfere with translation elongation or termination *in vivo* (35).

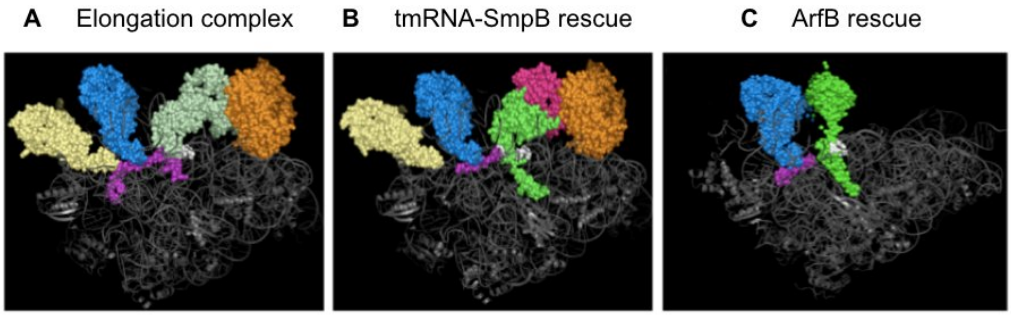

Figure **1-2**: Recognition of nonstop translation complexes. Structure model of an elongation complex (A) with an intact mRNA compared to recognition of nonstop translation complexes by tmRNA-SmpB (B) and ArfB (C). The 30S ribosomal subunits are shown in gray, with the decoding nucleotides G530, A1492, and A1493 in white. (A) An elongation complex trapped by kirromycin from PDB 2WRQ, with mRNA (purple), E-site tRNA (yellow), P-site tRNA (blue), and A-site tRNA (green) bound with EF-Tu (orange). (B) *trans*-Translation complex trapped by kirromycin from PDB 4ABR. The tRNA-like domain of tmRNA (pink) bound with EF-Tu (orange) is in a similar orientation to the acceptor stem of the tRNA in (A). SmpB (green) occupies the codon-anticodon region and extends into the empty mRNA channel. In nonstop translation complexes recognized by ArfB (C) (from PDB 4DH9), ArfB (green) extends into the empty mRNA channel, with the catalytic GGQ domain near the peptidyl-tRNA in the P site

(blue).

Whereas the crystal structure suggests that the mRNA channel downstream of the A site must be empty for tmRNA-SmpB to bind, kinetic data indicate that the mRNA channel does not always have to be empty for *trans*-translation to occur. The rate of *trans*- translation *in vitro* was measured using ribosomes stalled on mRNAs of different lengths (36). When the ribosomes were stalled with the mRNA channel completely occupied (with 15 nucleotides downstream of the P site), the reaction was extremely slow, consistent with the mRNA blocking tmRNA-SmpB. However, the reaction was rapid when the ribosomes were stalled with 0 to 6 nucleotides of mRNA downstream of the P site and was inhibited only partially with 9 to 12 nucleotides downstream of the P site. These results imply that mRNA in the A site, and even several codons downstream of the A site, does not interfere with *trans*- translation. The substrates used for the kinetic measurements were generated by omitting a tRNA from the reaction, so they probably do not occur frequently *in vivo*. However, the issue of whether tmRNA-SmpB can act on ribosomes with mRNA extending past the A site has important implications for the mechanism of *trans*-translation. It is possible that the interactions between SmpB and 16S rRNA observed in the crystal structure represent the lowest energy conformation, but these interactions are not required for tmRNA-SmpB to initiate *trans*-translation. Alternatively, when a ribosome stalls on an mRNA that does not completely fill the mRNA channel, it might undergo a structural change that allows SmpB access to the 16S rRNA. For example, the 3' end of the mRNA might loop out of the A site, or the ribosome could slide to the 3' end of the mRNA, leaving the A site empty. Such rearrangements could be facilitated by communication between the mRNA channel and the decoding center of the ribosome. Further biochemical experiments are required to determine whether *trans*-translation always requires an empty mRNA channel.

Substrates for *trans*-translation

Some of the known substrates for *trans*-translation are consistent with nonstop translation complexes generated by mRNA damage, but others suggest nonrandom or intentional mRNA cleavage to target translation reactions to *trans*-translation. Truncation of mRNA by premature termination of transcription, damage to the mRNA, or 3'-5' exonucleolytic mRNA turnover would be expected to be largely random and should produce nonstop translation complexes at a variety of positions along many mRNAs. Two proteomic-scale studies identified proteins tagged by *trans*-translation in *Caulobacter crescentus* and *Francisella tularensis*. Both studies found that many proteins are tagged and tagging occurs at locations throughout the protein sequence, as would be expected for activity on damaged mRNAs (37,38).

On the other hand, investigation of *E. coli* proteins that are tagged with high frequency indicates that there are some sequences prone to generate nonstop translation complexes (39). For example, in some substrates tagging occurs with high frequency after runs of rare codons or highly inefficient translation termination sequences (40-42). The mRNA is initially complete in these cases, but ribosome stalling during translation elongation or termination exposes the downstream mRNA to exonucleases, which chew back the mRNA to the leading edge of the ribosome to generate substrates for *trans*-translation (4,43-45). Exonuclease activity by RNase II can promote cleavage of the mRNA in the A site through an unknown mechanism, but RNase II and the corresponding A site cleavage are not essential for *trans*-translation on known substrates (46,47). Redundant nuclease activities may ensure that translation complexes stalled for an extended time are targeted for resolution by *trans*-translation.

In addition to ribosome stalling, errors during translation can lead to *trans*-translation. Suppressor tRNAs and drugs that promote miscoding increase the number of proteins tagged by *trans*-translation, demonstrating that read-through of the stop codon and frameshifting can result

in nonstop translation complexes when there is not an in-frame stop codon downstream (48,49). The examples described above all result in non-productive translation complexes, which could sequester ribosomes and limit new protein synthesis. The main purpose of *trans*-translation on these substrates is likely to be release of the ribosomes to maintain protein synthesis capacity.

There is also evidence that *trans*-translation is used to ensure the quality of the protein pool. *trans*-Translation increases on large proteins when *dnaK* is deleted, suggesting that misfolding of the nascent polypeptide might trigger mRNA cleavage to target the nascent polypeptide for proteolysis (50). It is now clear that interactions of the nascent chain in the peptide exit tunnel and communication between ribosome-associated chaperones and the catalytic center of the ribosome can affect the rate of translation (51,52). Terminally misfolded nascent proteins might be targeted to *trans*-translation to ensure they are rapidly degraded. It is not yet known whether there is a dedicated pathway for generating nonstop complexes that is triggered by misfolding, or whether misfolding slows elongation enough to expose the mRNA to nonspecific exonuclease activity.

Finally, *trans*-Translation is used intentionally as part of several regulatory circuits. RNase toxin components of toxin-antitoxin systems such as RelE and MazF cleave most mRNAs in the cell generating a large number of nonstop mRNAs and nonstop translation complexes (53,54). Toxin activity is used to induce stasis, allowing the cell to conserve resources during severe stress (53,54). Toxins are also activated in a small percentage of cells under optimal growth conditions to generate persister cells that can survive sudden stresses (55). *E. coli* mutants lacking *trans*-translation activity are defective in recovery from toxin-induced stasis, indicating that resolution of the nonstop complexes resulting from toxin activity is important for resuming growth after severe nutritional stress or persistence (56,57). Individual proteins are also targeted for *trans*-translation through truncation of the cognate mRNAs. Nuclease cleavage sites or transcriptional terminators 5' of the stop codon have been found in some *arfA* and *kinA* genes (58-60). Translation of these genes results in proteins that are rapidly degraded unless *trans*-translation is

impaired, making the encoded protein activity dependent on the state of *trans*-translation. The *arfA* example is described in more detail below. *trans*-Translation is used by LacI in *E. coli* to prevent excess protein accumulation (61). At high concentrations, LacI binds within the 3' end of its own gene. LacI binding to this site blocks transcription elongation and generates a nonstop mRNA, thereby targeting all newly expressed LacI for proteolysis. The use of *trans*-translation in regulatory circuits may be important for individual species or behaviors, but the evolutionary conservation of *trans*-translation is almost certainly due to the ability to maintain the protein synthesis capacity of the cell.

Physiology and alternatives to *trans*-translation

Genes encoding tmRNA (*ssrA*) and SmpB (*smpB*) have been identified in all sequenced bacterial species, including those with severely reduced genomes (62). This conservation suggests that *trans*-translation confers a selective advantage in all environments that support bacterial life. In fact, tmRNA and SmpB have been shown to be essential in several species, including *Neisseria gonorrhoeae, Shigella flexneri, Helicobacter pylori,* and *Mycobacterium tuberculosis* (63-66). Saturating genome-wide mutagenesis experiments suggest that tmRNA and SmpB are also required for viability in *Haemophilus influenza*, *Mycoplasma genitalium,* and *Staphylococcus aureus* (67-69). In other bacteria, tmRNA can be deleted with widely varying consequences. In some species, phenotypes of mutants lacking *trans*-translation activity are severe, including defects in virulence (*Salmonella enterica*, *Yersinia pestis, Francisella tularensis,* and *Streptococcus pneumoniae*), symbiosis (*Bradyrhizobium japonicum*), and cell cycle control (*C. crescentus*) (38,70-75). However, *E. coli* and *Bacillus subtilis* mutants that lack *trans*-translation have relatively mild phenotypes, such as increased antibiotic susceptibility and stress response

defects (48,76-78). Recent discoveries have shown that most or all species that do not require *trans*-translation have backup systems that resolve nonstop translation complexes when *trans*-translation activity is not available.

ArfA

On the basis of evolutionary conservation of *trans*-translation and the difference in phenotypes between *E. coli* and species in which tmRNA is essential, Chadani and coworkers performed a screen for genes that are essential in strains deleted for *ssrA* (79). They identified a single gene, *arfA,* and showed that the ArfA protein can promote hydrolysis of peptidyl-tRNA on nonstop translation complexes in an *in vitro* translation reaction. Release of the ribosomes by ArfA requires RF2, suggesting that ArfA recognizes the empty mRNA channel and recruits RF2 to hydrolyze the peptidyl-tRNA (Figure 1-3) (80). However, it is not yet clear how ArfA recognizes nonstop translation complexes. Hydroxyl radical probing experiments indicate that the N-terminal of ArfA binds near the decoding center and the C-terminus binds near the mRNA channel, even in the absence of RF2 (81). Binding of RF2 induces a conformational change in ArfA, so that the C-terminus of ArfA makes increased contacts with the mRNA channel (81). 21 nucleotides of mRNA extending past the P site do not inhibit ArfA-RF2 binding, but prevents conformational changes in ArfA, and mRNA with as few as 6 bases past the P site significantly inhibits peptidyl-tRNA hydrolysis (81-83). These data suggest a model whereby the C-terminus of ArfA senses mRNA channel occupancy and then allows RF2 to act as a codon-independent release factor (81).

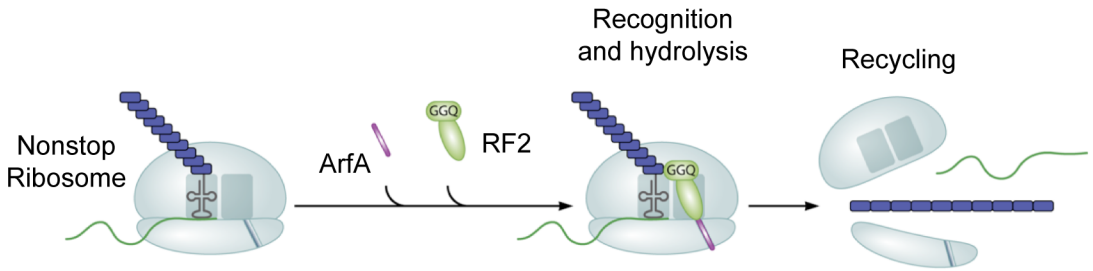

Figure **1-3**: Schematic of ArfA rescue. ArfA recruits RF2, which uses its GGQ motif to hydrolyze the peptidyl-tRNA in the ribosome. It is not known how ArfA recognizes nonstop translation complexes, but it might bind in the empty mRNA channel in a manner similar to SmpB

ArfA is a true backup system for *trans*-translation in that it is only active when *trans*-translation activity is not available. The *arfA* mRNA in *E. coli* includes a cleavage site for RNase III before the stop codon, and is efficiently cut by RNase III to produce a nonstop mRNA (58). Translation of *arfA* when *trans*-translation is active results in a tagged ArfA protein that is rapidly degraded. When *ssrA* is deleted, stable and active ArfA protein is produced. Presumably, regulation by *trans*-translation allows ArfA to release nonstop complexes only under physiological conditions where *trans*-translation is inactive or saturated. Most *arfA* genes from other species encode the RNase III cleavage site, but some use a transcriptional terminator before the stop codon to produce a nonstop mRNA (60). Thus, regulation of ArfA by *trans*-translation is conserved even though the mechanism for producing the nonstop mRNA is not.

Genetic experiments with *arfA* suggest that release of ribosomes from nonstop translation complexes is essential in *E. coli* and related species. In *E. coli,* deletion of *arfA* and *ssrA* is synthetically lethal (79). In contrast, *ssrA* is essential in *S. flexneri,* which does not have *arfA*, but *ssrA* can be deleted in *S. flexneri* cells that are engineered to express *E. coli arfA* (64). *arfA* genes have only been identified in a subset of beta- and gamma-proteobacteria and a handful of other species (60). However, the small size of *arfA* makes bioinformatic identification in distantly related bacteria difficult. The presence of *arfA* does not ensure that *trans*-translation is

dispensable. *N. gonorrhoeae* has an *arfA* gene, yet *trans*-translation is essential. The *N. gonorrhoeae arfA* is active when expressed in *E. coli* (60), so either *arfA* is not expressed in *N. gonorrhoeae* or its activity is not sufficient to support viability in the absence of *trans*-translation.

ArfB

ArfB was discovered in a multicopy suppressor screen for genes that allowed *E. coli* to survive without tmRNA or ArfA (Figure 1-4) (84). Peptidyl-tRNA hydrolase activity had been predicted for ArfB based on the presence of a GGQ motif common to release factors and peptidyl-tRNA hydrolases (85). In fact, purified ArfB specifically hydrolyzes peptidyl-tRNA in nonstop translation complexes *in vitro* (84,86). Structural studies show that ArfB recognizes nonstop complexes in a similar manner to SmpB-tmRNA: a C-terminal helix of ArfB extends into the empty mRNA channel and residues in this helix make contacts with 16S rRNA that are important for activity (Figure 1-2) (87,88). The physiological role of ArfB in *E. coli* is not clear. The chromosomal copy of a*rfB* will not support growth of *E. coli* in the absence of tmRNA and ArfA, and *ssrA* is essential in *S. flexneri* even though *arfB* is present (64,79). Either ArfB is reserved for special conditions in these species, or the availability of ArfA has made ArfB redundant and control of its expression has been lost. Chapter 2 of this work will show that ArfB in *Caulobacter crescentus* is functional in its chromosomal context and allows cells to survive without *trans*-translation. The *C. crescentus arfB* gene was identified in a Tn-seq experiment as a gene that is essential in cells lacking *ssrA* but not in wild type. ArfB homologs are widely distributed throughout bacteria. No regulation of ArfB by *trans*-translation has been identified, so unlike ArfA, ArfB may provide a constitutive, low level of resolution activity that only becomes significant when *trans*-translation is saturated or inactivated.

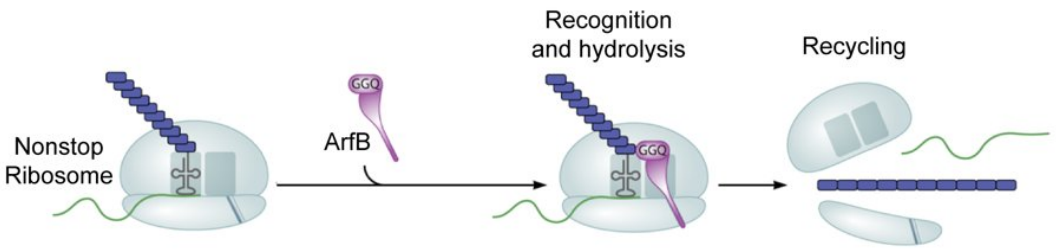

Figure **1-4**: Schematic of rescue by ArfB. ArfB encodes the conserved GGQ motif that is common to class I release factors. ArfB directly hydrolyzes peptidyl-tRNA on the ribosome, which then allows ribosome recycling.

The discoveries of ArfA and ArfB have important implications for understanding the role of *trans*-translation and the consequences of nonstop translation complexes. With the exception of *B. subtilis* and *F. tularensis,* all species in which *ssrA* or *smpB* have been deleted encode either ArfA or ArfB (Figure 1-5). Moreover, in all cases that have been tested, the ArfA or ArfB backup system becomes essential when *ssrA* is deleted. Therefore, at least one mechanism to resolve nonstop complexes may be required in most or all bacteria. Investigation of unknown alternative resolution mechanisms in *B. subtilis* is the subject of the Appendix. Some nonstop translation complexes may be resolved by "drop-off", dissociation of the peptidyl-tRNA from the ribosome followed by hydrolysis of the free peptidyl-tRNA by peptidyl-tRNA hydrolase (Pth). Drop-off occurs with some nascent chains of two to five amino acids, but longer chains have not been shown to dissociate without prior peptidyl-tRNA hydrolysis within the ribosome (89,90). Interactions between the nascent polypeptide and the exit channel may prevent drop-off in most cases. The discoveries of ArfA and ArfB make it clear that drop-off alone cannot support viability for most species in the absence of *trans*-translation.

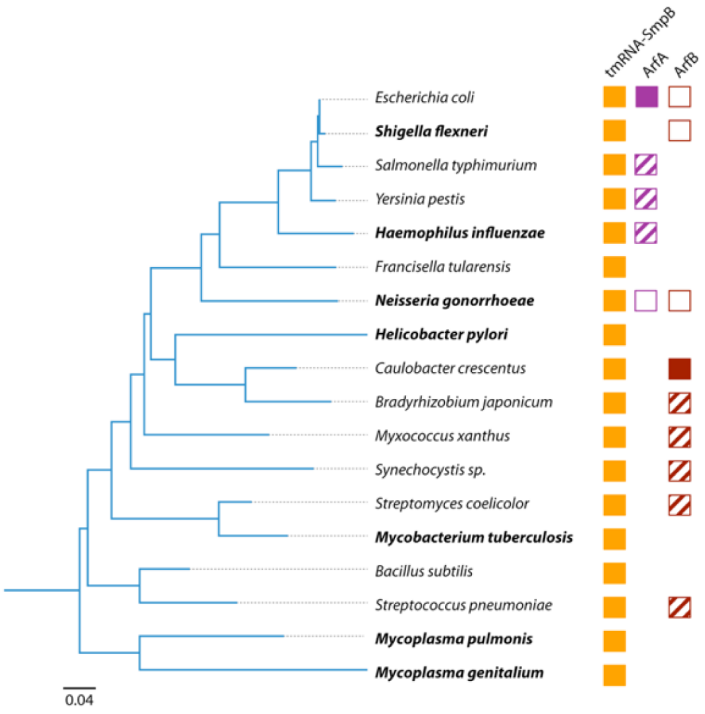

Figure **1-5**: Phylogenetic distribution of tmRNA-SmpB, ArfA, and ArfB. Species in which the phenotype of deleting *ssrA* or *smpB* is known are shown on a phylogenetic tree based on 16S rRNA sequences. Bold names indicate species in which *ssrA* or *smpB* is essential. The presence of genes encoding tmRNA-SmpB, ArfA, and ArfB is shown. For ArfA and ArfB, a filled box indicates that the system is sufficient to maintain viability in the absence of tmRNA-SmpB, an empty box indicates that the system is not sufficient to maintain viability in the absence of tmRNA-SmpB, and a hashed box indicates that it is not yet known whether the system is sufficient to maintain viability.

Why do all bacteria use *trans*-translation to resolve nonstop complexes, and some use only *trans*-translation, but none use only ArfA or ArfB? ArfA and ArfB do not completely mimic *trans*-translation, because they do not directly target the nascent polypeptide for proteolysis. Presumably, incomplete proteins released by ArfA or ArfB activity must be recognized and degraded by other proteolytic pathways in the cell. The fate of the mRNA during ArfA and ArfB activity is not yet known. It is likely that *trans*-translation is the preferred pathway because it

promotes degradation of the incomplete proteins and damaged mRNAs from nonstop complexes in addition to releasing the stalled ribosomes.

Targeting *trans*-translation for antibiotic development

The *trans*-translation pathway is an attractive target for development of new antibiotics because it is required for viability or virulence in many pathogenic strains and is not found in metazoans. Therefore, compounds that specifically inhibit *trans*-translation and not translation are likely to be effective for treating infections, yet have low toxicity on host cells. Compounds that inhibit *trans*-translation should kill *M. tuberculosis, N. gonorrhoeae, S. flexneri, H. influenza, S. aureus*, and other species in which *trans*-translation is essential, and could also prevent infection by *S. enterica, Y. pestis, F. tularensis, S. pneumonia*, and other species that require *trans*-translation for virulence. Compounds that inhibit ArfA and ArfB in addition to *trans*-translation may have antibacterial activity against all species.

Several cell-based assays for *trans*-translation activity have been described, and they have the same basic construction (91). A strong transcriptional terminator is inserted before the stop codon of a reporter gene, such as the *luc* gene encoding luciferase (Figure 1-6). Because the reporter protein is made from a nonstop mRNA, the protein is tagged and degraded if there is no inhibitor present. In the presence of an inhibitor, active reporter protein is produced. In principle, such assays could be used for screening any compound library for inhibitors.

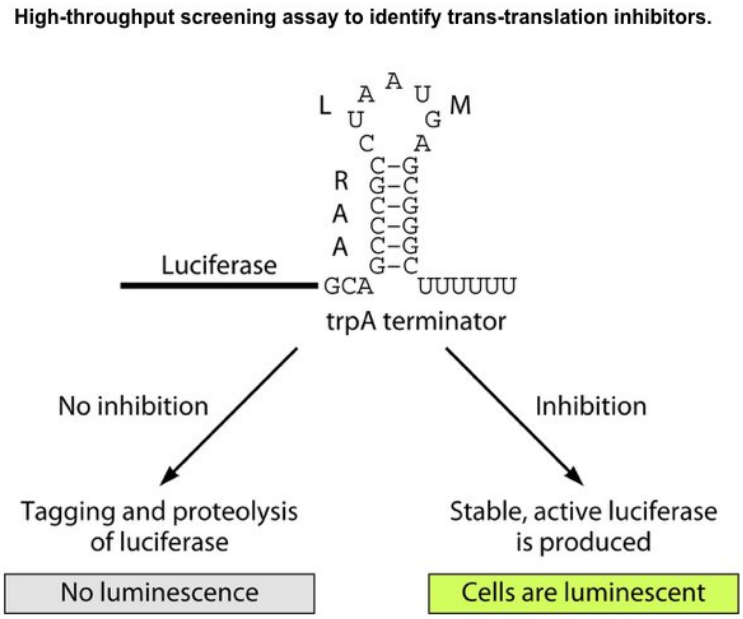

Figure **1-6**: High-throughput screening assay to identify *trans*-translation inhibitors. The reporter contains a gene encoding luciferase with a strong transcriptional terminator inserted before the stop codon, such that transcription results in a nonstop mRNA. *E. coli* cells containing the reporter were screened in high-throughput format to identify compounds that inhibit *trans*-translation. When no inhibitor is present, translation of the nonstop mRNA results in *trans*-translation followed by proteolysis of luciferase, and cells produce no luminescence. Conversely, active luciferase is produced when a *trans*-translation inhibitor is present, resulting in luminescence.

The results of one high-throughput screen for inhibiters of *trans*-translation have been reported (36,91). Several small molecules identified by HTS inhibit *trans*-translation but not translation *in vitro*. Growth inhibition assays with these compounds showed that they have broad-spectrum antibacterial activity (91). One compound, KKL-35, has a minimum inhibitory concentration of <2 µg/ml against pathogenic strains of *F. tularensis*, *Y. pestis, B. anthracis, B. mallei,* and *S. aureus* (91,92), (K. Keiler, unpublished). For KKL-35, growth inhibition of *E. coli*

was antagonized by low concentrations of puromycin, a drug that can release nonstop translation complexes by hydrolyzing peptidyl-tRNA on the ribosome (91). Likewise, growth inhibition of *S. flexneri* was antagonized by over-expression of *E. coli* ArfA. These results suggest that KKL-35 inhibits growth by preventing release of nonstop translation complexes. It has been suggested that translation inhibitors also inhibit cellular growth by jamming the essential SecY translocase and that jamming results in SecY degradation (93). Chapter 4 will examine whether KKL-35 also causes SecY degradation. Although many additional tests are required to determine if KKL-35 can be developed into a new antibiotic, it is clear that *trans*-translation and alternate pathways to resolve nonstop translation complexes are druggable. These pathways should be considered a prime target for further antibiotic development.

Ribosome rescue in eukaryotes

Resolution of nonstop translation complexes is just beginning to be characterized in eukaryotes, and the essentiality of ribosome rescue in mitochondria will be the subject of chapter 3. In eukaryotes translation of nuclear encoded genes occurs in the cytoplasm and mRNAs are capped and polyadenylated prior to export from the nucleus. Poly(A) binding protein interacts with translation initiation factors, which helps to ensure that truncated messages are not translated (1). However, polyadenylated nonstop mRNAs can arise from errors in transcription or aberrant processing of the 3' end prior to polyadenylation (94). Frameshifts during translation can also result in stop codon read through. When the ribosome translates a nonstop mRNA, it continues onto the poly(A) tract, encoding positively charged lysine residues that are thought to cause stalling by interacting with the negatively charged peptide exit channel (95-97). Translation is carried out in the cytoplasm and in mitochondria of eukaryotic cells, and each of these

compartments uses a different pathway to resolve nonstop translation complexes, which are described below.

DOM34:Hbs1

Nonstop translation complexes in the cytoplasm are resolved by DOM34 and Hbs1 in yeast or by Pelota and Hbs1 in mammals (95,98-100). DOM34 and Pelota have structural similarity to the eukaryotic release factor eRF1and Hbs1 is similar to the GTPase eRF3. In bacteria, rescue by either ArfA or ArfB results in hydrolysis of peptidyl-tRNA, followed by ribosome recycling. In contrast, the action of DOM34 and Hbs1 does not result in peptidyl-tRNA hydrolysis. Instead, DOM34 and Hbs1 dissociate the ribosomal subunits (100,101). In vitro, low levels of intact peptidyl-tRNA are released from the complex (100). However, in these experiments the ribosome was programmed with a short, 4 residue peptide, and further experiments are necessary to show whether a longer peptide could be efficiently released.

After subunit splitting by DOM34:Hbs1, components of the Ribosome Quality Control Complex (RQC) are recruited to the large subunit. The E3 ligase Ltn1p ubiquitylates the nascent peptide to target it for degradation, while Rqc2p facilitates the binding of $tRNA^{Ala}$ and $tRNA^{Thr}$ to the A site (102). The nascent peptide is transferred to these incoming tRNAs so that alanine and threonine residues are randomly incorporated at the C terminus of the peptide. These residues form a CAT tail (Carboxy terminus Alanine Threonine extension) (102). It has been proposed that accumulation of CAT tails trigger a heat shock response in the cell, but this response is only triggered in the absence of Ltn1p and it is unclear whether this response could occur in wild type cells where the ubiquitinated, CAT-tailed peptides are targeted for degradation (102,103). An alternative possibility is that the addition of A and T residues forces the peptide out of the exit

channel in order to expose more lysine residues (which have been encoded by the poly-A tail) to ubiquitination.

What is the fate of the large subunit and the peptidyl-tRNA that remains bound to it? It has been proposed that ubiquitination of the nascent chain targets both the aberrant peptide and the large subunit for degradation (104,105). However, it would be much more energetically favorable to hydrolyze the peptidyl- tRNA and preserve the large subunit. Perhaps an as yet unidentified factor can perform this function in the absence of the small subunit.

ICT1

Eukaryotic cells also synthesize protein in mitochondria. Mitochondria maintain a separate genome encoding components of the oxidative phosphorylation pathway and import all the necessary protein factors to carry out translation. Nonstop translation complexes are likely to be an even bigger problem in mitochondria because transcription and translation are performed in a single compartment as they are in bacteria. The absence of a physical barrier separating these processes means that the ribosome is likely to have access to mRNA as soon as it is transcribed, in contrast to cytoplasmic mRNAs which have undergone quality control prior to export from the nucleus. Additionally, pathways for degrading aberrant mRNA in mitochondria are likely much more limited than in the cytoplasm, although much more research in this area is needed (106).

An ArfB homolog, named ICT1 (88,107) is imported into the mammalian mitochondria. In chapter 3 of this dissertation, I will show that ICT1 hydrolyzes peptidyl-tRNA on nonstop translation complexes, and that the essential activity of ICT1 is ribosome rescue. ArfB and ICT1 both contain an N-terminal GGQ motif and a C-terminal $R(X_3)K(X_6)K(X_2)R$ motif that are required for peptidyl-tRNA hydrolase activity (88). tmRNA has been identified in organelles of some primitive eukaryotes, but is not retained in metazoans (62,108). It appears that most

eukaryotic mitochondria kept ArfB and dispensed with *trans*-translation, whereas bacteria retained *trans*-translation.

Summary

Nonstop mRNAs present a major challenge to all cells. The main pathways for rescuing ribosomes from nonstop mRNA are *trans*-translation (bacteria), ArfA (bacteria), DOM34:Hbs1 (eukaryotes) and ArfB (bacteria and eukaryotes). Broad conservation of this small number of rescue pathways suggests that they arose early in evolution and impart a significant selective advantage in all environments that support life on earth. Results presented in the following chapters will indicate that rescue of ribosomes from nonstop messages is essential in bacteria and in mitochondria of human cells.

References

1. Kahvejian A, Svitkin YV, Sukarieh R, M'Boutchou M-N, Sonenberg N. Mammalian poly(A)-binding protein is a eukaryotic translation initiation factor, which acts via multiple mechanisms. Genes Dev. Cold Spring Harbor Lab; 2005 Jan 1;19(1):104–13.

2. Doma MK, Parker R. RNA quality control in eukaryotes. Cell. 2007 Nov 16;131(4):660–8.

3. Laursen BS, Sørensen HP, Mortensen KK, Sperling-Petersen HU. Initiation of protein synthesis in bacteria. Microbiology and Molecular Biology Reviews. American Society for Microbiology; 2005 Mar;69(1):101–23.

4. Hayes CS, Sauer RT. Cleavage of the A site mRNA codon during ribosome pausing provides a mechanism for translational quality control. Mol Cell. 2003 Oct;12(4):903–11.

5. Sunohara T, Jojima K, Yamamoto Y, Inada T, Aiba H. Nascent-peptide-mediated ribosome stalling at a stop codon induces mRNA cleavage resulting in nonstop mRNA that is recognized by tmRNA. RNA. Cold Spring Harbor Laboratory Press; 2004 Mar;10(3):378–86.

6. Sunohara T, Jojima K, Tagami H, Inada T, Aiba H. Ribosome stalling during translation elongation induces cleavage of mRNA being translated in Escherichia coli. J Biol Chem. American Society for Biochemistry and Molecular Biology; 2004 Apr 9;279(15):15368–75.

7. Ito K, Chadani Y, Nakamori K, Chiba S, Akiyama Y, Abo T. Nascentome analysis uncovers futile protein synthesis in Escherichia coli. Spilianakis CB, editor. PLoS ONE. Public Library of Science; 2011;6(12):e28413.

8. Komine Y, Kitabatake M, Yokogawa T, Nishikawa K, Inokuchi H. A tRNA-like structure is present in 10Sa RNA, a small stable RNA from Escherichia coli. Proc Natl Acad Sci USA. National Academy of Sciences; 1994 Sep 27;91(20):9223–7.

9. Ushida C, Himeno H, Watanabe T, Muto A. tRNA-like structures in 10Sa RNAs of Mycoplasma capricolum and Bacillus subtilis. Nucleic Acids Research. Oxford University Press; 1994 Aug 25;22(16):3392–6.

10. Felden B, Himeno H, Muto A, McCutcheon JP, Atkins JF, Gesteland RF. Probing the structure of the Escherichia coli 10Sa RNA (tmRNA). RNA. Cold Spring Harbor Laboratory Press; 1997 Jan;3(1):89–103.

11. Tu GF, Reid GE, Zhang JG, Moritz RL, Simpson RJ. C-terminal extension of truncated recombinant proteins in Escherichia coli with a 10Sa RNA decapeptide. J Biol Chem. 1995 Apr 21;270(16):9322–6.

12. Williams KP, Bartel DP. Phylogenetic analysis of tmRNA secondary structure. RNA.

Cold Spring Harbor Laboratory Press; 1996 Dec;2(12):1306–10.

13. Karzai AW, Susskind MM, Sauer RT. SmpB, a unique RNA-binding protein essential for the peptide-tagging activity of SsrA (tmRNA). EMBO J. EMBO Press; 1999 Jul 1;18(13):3793–9.

14. Bessho Y, Shibata R, Sekine S-I, Murayama K, Higashijima K, Hori-Takemoto C, et al. Structural basis for functional mimicry of long-variable-arm tRNA by transfer-messenger RNA. Proc Natl Acad Sci USA. National Acad Sciences; 2007 May 15;104(20):8293–8.

15. Shimizu Y, Ueda T. SmpB triggers GTP hydrolysis of elongation factor Tu on ribosomes by compensating for the lack of codon-anticodon interaction during trans-translation initiation. J Biol Chem. 2006 Jun 9;281(23):15987–96.

16. Barends S, Wower J, Kraal B. Kinetic parameters for tmRNA binding to alanyl-tRNA synthetase and elongation factor Tu from Escherichia coli. Biochemistry. 2000 Mar 14;39(10):2652–8.

17. Rudinger-Thirion J, Giegé R, Felden B. Aminoacylated tmRNA from Escherichia coli interacts with prokaryotic elongation factor Tu. RNA. Cold Spring Harbor Laboratory Press; 1999 Aug;5(8):989–92.

18. Ivanova N, Pavlov MY, Bouakaz E, Ehrenberg M, Schiavone LH. Mapping the interaction of SmpB with ribosomes by footprinting of ribosomal RNA. Nucleic Acids Research. Oxford University Press; 2005;33(11):3529–39.

19. Kurita D, Sasaki R, Muto A, Himeno H. Interaction of SmpB with ribosome from directed hydroxyl radical probing. Nucleic Acids Research. Oxford University Press; 2007;35(21):7248–55.

20. Sundermeier TR, Karzai AW. Functional SmpB-ribosome interactions require tmRNA. J Biol Chem. American Society for Biochemistry and Molecular Biology; 2007 Nov 30;282(48):34779–86.

21. Valle M, Gillet R, Kaur S, Henne A, Ramakrishnan V, Frank J. Visualizing tmRNA entry into a stalled ribosome. Science. 2003 Apr 4;300(5616):127–30.

22. Ramrath DJF, Yamamoto H, Rother K, Wittek D, Pech M, Mielke T, et al. The complex of tmRNA-SmpB and EF-G on translocating ribosomes. Nature. Nature Publishing Group; 2012 May 24;485(7399):526–9.

23. Camenares D, Dulebohn DP, Svetlanov A, Karzai AW. Active and accurate trans-translation requires distinct determinants in the C-terminal tail of SmpB protein and the mRNA-like domain of transfer messenger RNA (tmRNA). J Biol Chem. American Society for Biochemistry and Molecular Biology; 2013 Oct 18;288(42):30527–42.

24. Keiler KC, Waller PR, Sauer RT. Role of a peptide tagging system in degradation of proteins synthesized from damaged messenger RNA. Science. 1996 Feb 16;271(5251):990–3.

25. Flynn JM, Levchenko I, Seidel M, Wickner SH, Sauer RT, Baker TA. Overlapping recognition determinants within the ssrA degradation tag allow modulation of proteolysis. Proc Natl Acad Sci USA. National Acad Sciences; 2001 Sep 11;98(19):10584–9.

26. Gottesman S, Roche E, Zhou Y, Sauer RT. The ClpXP and ClpAP proteases degrade proteins with carboxy-terminal peptide tails added by the SsrA-tagging system. Genes Dev. Cold Spring Harbor Laboratory Press; 1998 May 1;12(9):1338–47.

27. Herman C, Thévenet D, Bouloc P, Walker GC, D'Ari R. Degradation of carboxy-terminal-tagged cytoplasmic proteins by the Escherichia coli protease HflB (FtsH). Genes Dev. Cold Spring Harbor Laboratory Press; 1998 May 1;12(9):1348–55.

28. Choy JS, Aung LL, Karzai AW. Lon protease degrades transfer-messenger RNA-tagged proteins. Journal of Bacteriology. American Society for Microbiology; 2007 Sep;189(18):6564–71.

29. Richards J, Mehta P, Karzai AW. RNase R degrades nonstop mRNAs selectively in an SmpB-tmRNA-dependent manner. Molecular Microbiology. Blackwell Publishing Ltd; 2006 Dec;62(6):1700–12.

30. Mehta P, Richards J, Karzai AW. tmRNA determinants required for facilitating nonstop mRNA decay. RNA. Cold Spring Harbor Lab; 2006 Dec;12(12):2187–98.

31. Yamamoto Y, Sunohara T, Jojima K, Inada T, Aiba H. SsrA-mediated trans-translation plays a role in mRNA quality control by facilitating degradation of truncated mRNAs. RNA. Cold Spring Harbor Laboratory Press; 2003 Apr;9(4):408–18.

32. Neubauer C, Gillet R, Kelley AC, Ramakrishnan V. Decoding in the absence of a codon by tmRNA and SmpB in the ribosome. Science. American Association for the Advancement of Science; 2012 Mar 16;335(6074):1366–9.

33. Sundermeier TR, Dulebohn DP, Cho HJ, Karzai AW. A previously uncharacterized role for small protein B (SmpB) in transfer messenger RNA-mediated trans-translation. Proc Natl Acad Sci USA. National Acad Sciences; 2005 Feb 15;102(7):2316–21.

34. Kurita D, Muto A, Himeno H. Role of the C-terminal tail of SmpB in the early stage of trans-translation. RNA. Cold Spring Harbor Lab; 2010 May;16(5):980–90.

35. Moore SD, Sauer RT. Ribosome rescue: tmRNA tagging activity and capacity in Escherichia coli. Molecular Microbiology. Blackwell Science Ltd; 2005 Oct;58(2):456–66.

36. Ivanova N, Pavlov MY, Felden B, Ehrenberg M. Ribosome rescue by tmRNA requires truncated mRNAs. J Mol Biol. 2004 Apr 16;338(1):33–41.

37. Hong S-J, Lessner FH, Mahen EM, Keiler KC. Proteomic identification of tmRNA substrates. Proc Natl Acad Sci USA. National Acad Sciences; 2007 Oct 23;104(43):17128–33.

38. Svetlanov A, Puri N, Mena P, Koller A, Karzai AW. Francisella tularensis tmRNA system mutants are vulnerable to stress, avirulent in mice, and provide effective immune protection. Molecular Microbiology. Blackwell Publishing Ltd; 2012 Jul;85(1):122–41.

39. Roche ED, Sauer RT. Identification of endogenous SsrA-tagged proteins reveals tagging at positions corresponding to stop codons. J Biol Chem. American Society for Biochemistry and Molecular Biology; 2001 Jul 27;276(30):28509–15.

40. Roche ED, Sauer RT. SsrA-mediated peptide tagging caused by rare codons and tRNA scarcity. EMBO J. EMBO Press; 1999 Aug 16;18(16):4579–89.

41. Hayes CS, Bose B, Sauer RT. Proline residues at the C terminus of nascent chains induce SsrA tagging during translation termination. J Biol Chem. American Society for Biochemistry and Molecular Biology; 2002 Sep 13;277(37):33825–32.

42. Hayes CS, Bose B, Sauer RT. Stop codons preceded by rare arginine codons are efficient determinants of SsrA tagging in Escherichia coli. Proc Natl Acad Sci USA. National Acad Sciences; 2002 Mar 19;99(6):3440–5.

43. Li X, Yokota T, Ito K, Nakamura Y, Aiba H. Reduced action of polypeptide release factors induces mRNA cleavage and tmRNA tagging at stop codons in Escherichia coli. Molecular Microbiology. Blackwell Publishing Ltd; 2007 Jan;63(1):116–26.

44. Li X, Hirano R, Tagami H, Aiba H. Protein tagging at rare codons is caused by tmRNA action at the 3' end of nonstop mRNA generated in response to ribosome stalling. RNA. Cold Spring Harbor Lab; 2006 Feb;12(2):248–55.

45. Garza-Sánchez F, Gin JG, Hayes CS. Amino acid starvation and colicin D treatment induce A-site mRNA cleavage in Escherichia coli. J Mol Biol. 2008 May 2;378(3):505–19.

46. Garza-Sánchez F, Shoji S, Fredrick K, Hayes CS. RNase II is important for A-site mRNA cleavage during ribosome pausing. Molecular Microbiology. Blackwell Publishing Ltd; 2009 Sep;73(5):882–97.

47. Janssen BD, Garza-Sánchez F, Hayes CS. A-site mRNA cleavage is not required for tmRNA-mediated ssrA-peptide tagging. Herman C, College of Medicine United States of America B, editors. PLoS ONE. Public Library of Science; 2013;8(11):e81319.

48. Abo T, Ueda K, Sunohara T, Ogawa K, Aiba H. SsrA-mediated protein tagging in the presence of miscoding drugs and its physiological role in Escherichia coli. Genes Cells. 2002 Jul;7(7):629–38.

49. Ueda K, Yamamoto Y, Ogawa K, Abo T, Inokuchi H, Aiba H. Bacterial SsrA system plays a role in coping with unwanted translational readthrough caused by suppressor tRNAs. Genes Cells. 2002 May;7(5):509–19.

50. Ruhe ZC, Hayes CS. The N-terminus of GalE induces tmRNA activity in Escherichia coli. Kudla G, editor. PLoS ONE. Public Library of Science; 2010;5(12):e15207.

51. Kramer G, Boehringer D, Ban N, Bukau B. The ribosome as a platform for co-translational processing, folding and targeting of newly synthesized proteins. Nature Structural & Molecular Biology. Nature Publishing Group; 2009 Jun;16(6):589–97.

52. Ito K, Chiba S. Arrest peptides: cis-acting modulators of translation. Annu Rev Biochem. Annual Reviews; 2013;82(1):171–202.

53. Pedersen K, Zavialov AV, Pavlov MY, Elf J, Gerdes K, Ehrenberg M. The bacterial toxin RelE displays codon-specific cleavage of mRNAs in the ribosomal A site. Cell. 2003 Jan 10;112(1):131–40.

54. Zhang Y, Zhang J, Hoeflich KP, Ikura M, Qing G, Inouye M. MazF cleaves cellular mRNAs specifically at ACA to block protein synthesis in Escherichia coli. Mol Cell. 2003 Oct;12(4):913–23.

55. Maisonneuve E, Shakespeare LJ, Jørgensen MG, Gerdes K. Bacterial persistence by RNA endonucleases. Proc Natl Acad Sci USA. National Acad Sciences; 2011 Aug 9;108(32):13206–11.

56. Christensen SK, Gerdes K. RelE toxins from bacteria and Archaea cleave mRNAs on translating ribosomes, which are rescued by tmRNA. Molecular Microbiology. 2003 Jun;48(5):1389–400.

57. Christensen SK, Pedersen K, Hansen FG, Gerdes K. Toxin-antitoxin loci as stress-response-elements: ChpAK/MazF and ChpBK cleave translated RNAs and are counteracted by tmRNA. J Mol Biol. 2003 Sep 26;332(4):809–19.

58. Garza-Sánchez F, Schaub RE, Janssen BD, Hayes CS. tmRNA regulates synthesis of the ArfA ribosome rescue factor. Molecular Microbiology. Blackwell Publishing Ltd; 2011 Jun;80(5):1204–19.

59. Kobayashi K, Kuwana R, Takamatsu H. kinA mRNA is missing a stop codon in the undomesticated Bacillus subtilis strain ATCC 6051. Microbiology (Reading, Engl). Microbiology Society; 2008 Jan;154(Pt 1):54–63.

60. Schaub RE, Poole SJ, Garza-Sánchez F, Benbow S, Hayes CS. Proteobacterial ArfA peptides are synthesized from nonstop messenger RNAs. J Biol Chem. American Society for Biochemistry and Molecular Biology; 2012 Aug 24;287(35):29765–75.

61. Abo T, Inada T, Ogawa K, Aiba H. SsrA-mediated tagging and proteolysis of LacI and its role in the regulation of lac operon. EMBO J. EMBO Press; 2000 Jul 17;19(14):3762–9.

62. Gueneau de Novoa P, Williams KP. The tmRNA website: reductive evolution of tmRNA in plastids and other endosymbionts. Nucleic Acids Research. Oxford University Press; 2004 Jan 1;32(Database issue):D104–8.

63. Huang C, Wolfgang MC, Withey J, Koomey M, Friedman DI. Charged tmRNA but not tmRNA-mediated proteolysis is essential for Neisseria gonorrhoeae viability. EMBO J. EMBO Press; 2000 Mar 1;19(5):1098–107.

64. Ramadoss NS, Zhou X, Keiler KC. tmRNA is essential in Shigella flexneri. Herman C, editor. PLoS ONE. Public Library of Science; 2013;8(2):e57537.

65. Personne Y, Parish T. Mycobacterium tuberculosis possesses an unusual tmRNA rescue system. Tuberculosis (Edinb). 2014 Jan;94(1):34–42.

66. Thibonnier M, Thiberge J-M, De Reuse H. Trans-translation in Helicobacter pylori: essentiality of ribosome rescue and requirement of protein tagging for stress resistance and competence. Ahmed N, editor. PLoS ONE. Public Library of Science; 2008;3(11):e3810.

67. Fey PD, Endres JL, Yajjala VK, Widhelm TJ, Boissy RJ, Bose JL, et al. A genetic resource for rapid and comprehensive phenotype screening of nonessential Staphylococcus aureus genes. MBio. American Society for Microbiology; 2013;4(1):e00537–12.

68. Chaudhuri RR, Allen AG, Owen PJ, Shalom G, Stone K, Harrison M, et al. Comprehensive identification of essential Staphylococcus aureus genes using Transposon-Mediated Differential Hybridisation (TMDH). BMC Genomics. BioMed Central; 2009;10(1):291.

69. Glass JI, Assad-Garcia N, Alperovich N, Yooseph S, Lewis MR, Maruf M, et al. Essential genes of a minimal bacterium. Proc Natl Acad Sci USA. National Acad Sciences; 2006 Jan 10;103(2):425–30.

70. Julio SM, Heithoff DM, Mahan MJ. ssrA (tmRNA) plays a role in Salmonella enterica serovar Typhimurium pathogenesis. Journal of Bacteriology. American Society for Microbiology (ASM); 2000 Mar;182(6):1558–63.

71. Bäumler AJ, Kusters JG, Stojiljkovic I, Heffron F. Salmonella typhimurium loci involved in survival within macrophages. Infect Immun. American Society for Microbiology (ASM); 1994 May;62(5):1623–30.

72. Okan NA, Mena P, Benach JL, Bliska JB, Karzai AW. The smpB-ssrA mutant of Yersinia pestis functions as a live attenuated vaccine to protect mice against pulmonary plague infection. Infect Immun. American Society for Microbiology; 2010 Mar;78(3):1284–93.

73. Keiler KC, Shapiro L. TmRNA is required for correct timing of DNA replication in Caulobacter crescentus. Journal of Bacteriology. American Society for Microbiology (ASM); 2003 Jan;185(2):573–80.

74. Ebeling S, Kündig C, Hennecke H. Discovery of a rhizobial RNA that is essential for symbiotic root nodule development. Journal of Bacteriology. American Society for Microbiology (ASM); 1991 Oct;173(20):6373–82.

75. Mann B, van Opijnen T, Wang J, Obert C, Wang Y-D, Carter R, et al. Control of virulence by small RNAs in Streptococcus pneumoniae. Cossart P, editor. PLoS Pathog. Public Library of Science; 2012;8(7):e1002788.

76. Muto A, Fujihara A, Ito KI, Matsuno J, Ushida C, Himeno H. Requirement of transfer-messenger RNA for the growth of Bacillus subtilis under stresses. Genes Cells. 2000 Aug;5(8):627–35.

77. Shin J-H, Price CW. The SsrA-SmpB ribosome rescue system is important for growth of Bacillus subtilis at low and high temperatures. Journal of Bacteriology. American Society for Microbiology; 2007 May;189(10):3729–37.

78. Munavar H, Zhou Y, Gottesman S. Analysis of the Escherichia coli Alp phenotype: heat shock induction in ssrA mutants. Journal of Bacteriology. American Society for Microbiology; 2005 Jul;187(14):4739–51.

79. Chadani Y, Ono K, Ozawa S-I, Takahashi Y, Takai K, Nanamiya H, et al. Ribosome rescue by Escherichia coli ArfA (YhdL) in the absence of trans-translation system. Molecular Microbiology. Blackwell Publishing Ltd; 2010 Nov;78(4):796–808.

80. Chadani Y, Ito K, Kutsukake K, Abo T. ArfA recruits release factor 2 to rescue stalled ribosomes by peptidyl-tRNA hydrolysis in Escherichia coli. Molecular Microbiology. Blackwell Publishing Ltd; 2012 Oct;86(1):37–50.

81. Kurita D, Chadani Y, Muto A, Abo T, Himeno H. ArfA recognizes the lack of mRNA in the mRNA channel after RF2 binding for ribosome rescue. Nucleic Acids Research. Oxford University Press; 2014 Dec 1;42(21):13339–52.

82. Shimizu Y. ArfA recruits RF2 into stalled ribosomes. J Mol Biol. 2012 Nov 2;423(4):624–31.

83. Zeng F, Jin H. Peptide release promoted by methylated RF2 and ArfA in nonstop translation is achieved by an induced-fit mechanism. RNA. Cold Spring Harbor Lab; 2016 Jan;22(1):49–60.

84. Chadani Y, Ono K, Kutsukake K, Abo T. Escherichia coli YaeJ protein mediates a novel ribosome-rescue pathway distinct from SsrA- and ArfA-mediated pathways. Molecular Microbiology. Blackwell Publishing Ltd; 2011 May;80(3):772–85.

85. Hayes CS, Keiler KC. Beyond ribosome rescue: tmRNA and co-translational processes. FEBS Lett. 2010 Jan 21;584(2):413–9.

86. Handa Y, Inaho N, Nameki N. YaeJ is a novel ribosome-associated protein in Escherichia coli that can hydrolyze peptidyl-tRNA on stalled ribosomes. Nucleic Acids Research. 2011 Mar 19;39(5):1739–48.

87. Gagnon MG, Seetharaman SV, Bulkley D, Steitz TA. Structural basis for the rescue of stalled ribosomes: structure of YaeJ bound to the ribosome. Science. American Association for the Advancement of Science; 2012 Mar 16;335(6074):1370–2.

88. Kogure H, Handa Y, Nagata M, Kanai N, Guntert P, Kubota K, et al. Identification of residues required for stalled-ribosome rescue in the codon-independent release factor YaeJ. Nucleic Acids Research. 2014 Mar 11;42(5):3152–63.

89. Cruz-Vera LR, Magos-Castro MA, Zamora-Romo E, Guarneros G. Ribosome stalling and peptidyl-tRNA drop-off during translational delay at AGA codons. Nucleic Acids Research. Oxford University Press; 2004;32(15):4462–8.

90. Gonzalez de Valdivia EI, Isaksson LA. Abortive translation caused by peptidyl-tRNA drop-off at NGG codons in the early coding region of mRNA. FEBS J. Blackwell Science Ltd; 2005 Oct;272(20):5306–16.

91. Ramadoss NS, Alumasa JN, Cheng L, Wang Y, Li S, Chambers BS, et al. Small molecule inhibitors of trans-translation have broad-spectrum antibiotic activity. Proc Natl Acad Sci USA. National Acad Sciences; 2013 Jun 18;110(25):10282–7.

92. Goralski TDP, Dewan KK, Alumasa JN, Avanzato V, Place DE, Markley RL, et al. Inhibitors of ribosome rescue arrest growth of Francisella tularensis at all stages of intracellular replication. Antimicrob Agents Chemother. American Society for Microbiology; 2016 Mar 7;:AAC.03089–15.

93. van Stelten J, Silva F, Belin D, Silhavy TJ. Effects of antibiotics and a proto-oncogene homolog on destruction of protein translocator SecY. Science. American Association for the Advancement of Science; 2009 Aug 7;325(5941):753–6.

94. Wilson MA, Meaux S, van Hoof A. A genomic screen in yeast reveals novel aspects of nonstop mRNA metabolism. Genetics. Genetics; 2007 Oct;177(2):773–84.

95. Shoemaker CJ, Green R. Translation drives mRNA quality control. Nature Structural & Molecular Biology. Nature Publishing Group; 2012 Jun;19(6):594–601.

96. Koutmou KS, Schuller AP, Brunelle JL, Radhakrishnan A, Djuranovic S, Green R. Ribosomes slide on lysine-encoding homopolymeric A stretches. Elife. eLife Sciences Publications Limited; 2015;4:446.

97. Choe Y-J, Park S-H, Hassemer T, Körner R, Vincenz-Donnelly L, Hayer-Hartl M, et al. Failure of RQC machinery causes protein aggregation and proteotoxic stress. Nature. Nature Publishing Group; 2016 Mar 10;531(7593):191–5.

98. Doma MK, Parker R. Endonucleolytic cleavage of eukaryotic mRNAs with stalls in translation elongation. Nature. Nature Publishing Group; 2006 Mar 23;440(7083):561–4.

99. Passos DO, Doma MK, Shoemaker CJ, Muhlrad D, Green R, Weissman J, et al. Analysis of Dom34 and its function in no-go decay. Mol Biol Cell. American Society for Cell Biology; 2009 Jul;20(13):3025–32.

100. Pisareva VP, Skabkin MA, Hellen CUT, Pestova TV, Pisarev AV. Dissociation by Pelota, Hbs1 and ABCE1 of mammalian vacant 80S ribosomes and stalled elongation complexes. EMBO J. EMBO Press; 2011 May 4;30(9):1804–17.

101. van den Elzen AMG, Schuller A, Green R, Séraphin B. Dom34-Hbs1 mediated dissociation of inactive 80S ribosomes promotes restart of translation after stress. EMBO J. EMBO Press; 2014 Feb 3;33(3):265–76.

102. Shen PS, Park J, Qin Y, Li X, Parsawar K, Larson MH, et al. Protein synthesis. Rqc2p and 60S ribosomal subunits mediate mRNA-independent elongation of nascent chains. Science. American Association for the Advancement of Science; 2015 Jan 2;347(6217):75–8.

103. Brandman O, Stewart-Ornstein J, Wong D, Larson A, Williams CC, Li G-W, et al. A ribosome-bound quality control complex triggers degradation of nascent peptides and signals translation stress. Cell. 2012 Nov 21;151(5):1042–54.

104. Fujii K, Kitabatake M, Sakata T, Ohno M. 40S subunit dissociation and proteasome-dependent RNA degradation in nonfunctional 25S rRNA decay. EMBO J. EMBO Press; 2012 May 30;31(11):2579–89.

105. Defenouillere Q, Yao Y, Mouaikel J, Namane A, Galopier A, Decourty L, et al. Cdc48-associated complex bound to 60S particles is required for the clearance of aberrant translation products. Proc Natl Acad Sci USA. National Acad Sciences; 2013 Mar 26;110(13):5046–51.

106. Borowski LS, Szczesny RJ, Brzezniak LK, Stepien PP. RNA turnover in human mitochondria: more questions than answers? Biochim Biophys Acta. 2010 Jun;1797(6-7):1066–70.

107. Richter R, Rorbach J, Pajak A, Smith PM, Wessels HJ, Huynen MA, et al. A functional peptidyl-tRNA hydrolase, ICT1, has been recruited into the human mitochondrial ribosome. EMBO J. 2010 Feb 25;29(6):1116–25.

108. Keiler KC, Shapiro L, Williams KP. tmRNAs that encode proteolysis-inducing tags are found in all known bacterial genomes: A two-piece tmRNA functions in Caulobacter. Proc Natl Acad Sci USA. National Academy of Sciences; 2000 Jul 5;97(14):7778–83.

Chapter 2

Release of nonstop ribosomes is essential in bacteria

Adapted from:

Feaga HA, Viollier PH, Keiler KC. 2014. Release of nonstop ribosomes is essential. *mBio*. 5:e01916-14. doi:10.1128/mBio.01916-14.

Abstract

Bacterial ribosomes frequently translate to the 3' end of an mRNA without terminating at a stop codon. Almost all bacteria use the tmRNA-based *trans*-translation pathway to release these "non-stop" ribosomes and maintain protein synthesis capacity. *trans*-Translation is essential in some species, but in others, such as *Caulobacter crescentus*, *trans*-translation can be inactivated. To determine why *trans*-translation is dispensable in *C. crescentus,* a Tn-seq screen was used to identify genes that specifically alter growth in cells lacking *ssrA*, the gene encoding tmRNA. One of these genes, *CC1214*, was essential in Δ*ssrA* cells. Purified CC1214 protein could release non-stop ribosomes in vitro. CC1214 is a homolog of the *Escherichia coli* ArfB protein, and using the CC1214 sequence ArfB homologs were identified in the majority of bacterial phyla. Most species in which *ssrA* has been deleted contain an ArfB homolog, suggesting that release of non-stop ribosomes may be essential in most or all bacteria.

Introduction

The efficient translation cycle of initiation, elongation, termination, and recycling, allows rapid protein synthesis and cellular growth. Most ribosomes pass through this cycle many times every cell generation, so breaks in the cycle can lead to rapid depletion of protein synthesis capacity. A major source of translation stress in bacteria is truncated mRNA. Because bacterial ribosomes do not require any information from the 3' end of an mRNA for initiation, they can start translating mRNAs that are incompletely transcribed or damaged (1). If the mRNA does not have a stop codon, or if the ribosome reads through the stop codon and reaches the 3' end of the mRNA without terminating, the translation cycle will be broken. A stop codon in the decoding center is required for release factors RF1 or RF2 to hydrolyze the peptidyl-tRNA and allow the ribosome to be recycled (2). When the ribosome is at the 3' end of the mRNA but there is no stop codon in the decoding center, *trans*-translation is used to resolve the "non-stop" translation complex and allow the translation cycle to resume (3). During *trans*-translation, tmRNA, encoded by the *ssrA* gene, and SmpB, a small protein, recognize a non-stop translation complex and divert the ribosome onto a reading frame within tmRNA. A stop codon at the end of the tmRNA reading frame allows translation termination and ribosome recycling. *trans*-Translation also targets the nascent polypeptide and problematic mRNA for rapid degradation, clearing the cell of all components of the non-stop translation complex (4-7).

Genes encoding tmRNA and SmpB are found in >99% of sequenced bacterial genomes (8), suggesting that *trans*-translation confers a selective advantage under most environmental conditions. In culture, *trans*-translation is essential in some species. However, *ssrA* can be deleted in some bacteria without causing a severe phenotype (9).

The apparent contradiction between the universal conservation of *trans*-translation and the mild phenotype caused by removing this system in *E. coli* was explained by the discovery of

ArfA (alternative rescue factor A). The *arfA* gene was identified in a screen for mutants that were synthetically lethal with an *ssrA* deletion (10). ArfA is a small protein that allows RF2 to hydrolyze peptidyl-tRNA on non-stop translation complexes (11,12). ArfA is a true backup system for *trans*-translation, because ArfA itself is made from a truncated mRNA (13,14). When *trans*-translation is functional, ArfA is tagged by *trans*-translation and degraded. If *trans*-translation is disabled or overwhelmed, untagged ArfA is produced to release the accumulating non-stop translation complexes. ArfA activity allows *E. coli* to live without *trans*-translation, but in Δ*arfA* cells *ssrA* is essential (10). Likewise, *ssrA* is essential in *S. flexneri*, which does not have *arfA*, but *ssrA* can be deleted in *S. flexneri* expressing the *E. coli arfA* gene (15). *arfA* genes have been identified in many γ-proteobacteria and some β-proteobacteria, and may explain why *ssrA* can be deleted in species such as *Salmonella enterica* and *Yersinia pestis* (9,10,13). Other bacteria, such as *Caulobacter crescentus*, do not have an *arfA* homolog but still do not require *trans*-translation for viability.

This chapter describes the results of a Tn-Seq screen to identify genetic interactions with *ssrA* in *C. crescentus*. One gene, *CC1214*, was shown to be essential in Δ*ssrA* cells but not in wild-type cells. The CC1214 protein could hydrolyze peptidyl-tRNA in non-stop translation complexes in vitro, and had homology to *E. coli* ArfB. The phylogenetic distribution of CC1214 homologs raises the possibility that all or most bacteria require at least one system to resolve non-stop translation complexes.

Results

Identification of genetic interactions with *ssrA*

Genetic interactions with *ssrA* were probed using transposon mutagenesis followed by sequencing (Tn-seq) (16-20). Wild-type and Δ*ssrA* cultures were individually mutagenized with the *himar1* transposon and plated on selective medium to allow growth of cells with an integrated transposon. Colonies were pooled and sequenced to identify the location of transposon insertions. In this experiment, the number of times each sequence is recovered depends on the frequency of transposon insertion at that site and the amount of colony grown from the mutant cells. Because the frequency of transposition at each site is a function of the DNA sequence and chromosomal context, which are the same in both strains, the ratio of the number of times a sequence is recovered in Δ*ssrA* and wild-type cultures provides a measure of the relative fitness of the mutant in each strain background. Mutants with a low ratio are relatively more fit in wild type, whereas mutants with a high ratio are relatively more fit in Δ*ssrA* cells. Sequence data were pooled to generate a ratio for each gene (Table 2-1).

Table 2-1 Selected genes identified by Tn-seq

gene	insertions in Δ*ssrA*[1]	insertions in wt[1]	$\log_{10}$ratio[2]
CC1214	1	564	-2.45
$tRNA^{Arg}$ (CCNA R0010)	0	214	-2.33
rluD	39	2930	-1.87
lepA	90	5478	-1.78
efp	3	139	-1.54
pth	0	24	-1.39
CC1215	85851	1011	1.93
smpB	1644	38	1.62

[1]Number of insertions, normalized to the total number of reads in the larger dataset. [2]Ratio as

calculated after adding 1 to the number of insertions to eliminate infinite and zero ratios.

Genes with a low ratio include *rluD*, *lepA, efp, pth*, and $tRNA^{Arg}$(CCU), which have genetic or functional interactions with *trans*-translation in *E. coli*. Deletion of *rluD* increases the frequency of stop codon readthrough and increases the tagging of *E. coli* proteins (21). LepA (EF4) is a translation elongation factor, and over-expression of *lepA* inhibits *trans*-translation (22). EF-P promotes translation through polyproline sequences and other sequences that stall translation elongation (23). Deletion of *rluD, lepA,* or *efp* is likely to generate more substrates for *trans*-translation, so it is not unexpected that mutations in these genes would have a synthetic phenotype in the Δ*ssrA* background. Multiple genetic interactions between *pth* and *ssrA* have been reported in *E. coli*, including exacerbation of *pth* mutant phenotypes in Δ*ssrA* cells, so it is not surprising that mutation of *pth* in *C. crescentus* Δ*ssrA* cells results in relatively lower fitness (24). $tRNA^{Arg}$(CCU) is a rare tRNA, and the amount of *trans*-translation of genes with a AGG codon in *E. coli* is negatively correlated with the abundance of $tRNA^{Arg}$(CCU) (25).

CC1214, annotated as a peptidyl-tRNA hydrolase family protein, had the lowest ratio of any gene (Figure 2-1, Table 2-1). Sequence homology searches suggested that *CC1214* encoded a homolog of *E. coli* ArfB, a gene identified as a multicopy suppressor of lethality in *E. coli* lacking both *ssrA* and *arfA* (26). *E. coli arfB* cannot support viability from its chromosomal context (10). Nevertheless, the sequence similarity to ArfB made CC1214 a candidate for releasing non-stop complexes in the absence of *trans*-translation in *C. crescentus*.

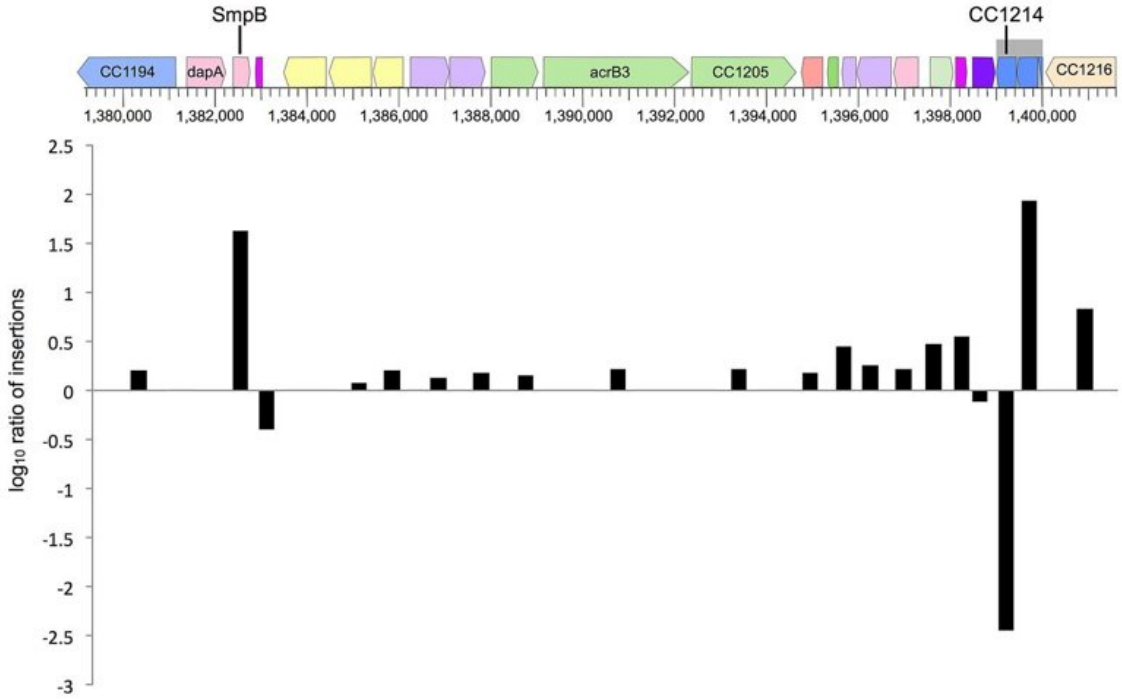

Figure **2-1**: Tn-seq identification of genes interacting with *ssrA*. After Tn-seq, the $\log_{10}$ ratio of the normalized number of transposon insertions in Δ*ssrA* cells to the number in wild-type cells was calculated and plotted for a portion of the *C. crescentus* chromosome. The organization of genes and the direction of transcription are represented with colored arrows.

Genes with a high ratio include *smpB* and *CC1215* (Table 2-1). Deletion of *smpB* in wild-type cells causes slow growth, but deletion of *smpB* in Δ*ssrA* cells does not cause any additional growth defect, resulting in a relatively higher fitness in the Δ*ssrA* background. Because a large number of insertions in *CC1215* were recovered in both strains, the high ratio for *CC1215* is likely due to increased fitness in Δ*ssrA* cells. Because *CC1214* and *CC1215* are in an operon, the interactions of both these genes with *ssrA* were investigated.

***CC1214* deletion is synthetically lethal with *ssrA* deletion**

The Tn-seq data suggest that cells lacking both *trans*-translation and *CC1214* are at a severe competitive disadvantage. To characterize the phenotype of cells lacking *CC1214,* the gene was replaced with an Ω cassette encoding spectinomycin resistance using 2-step recombination (27). The *CC1214::Ω* strain grew at the same rate as wild type (Table 2-2), and showed no morphological defects. In contrast, *CC1214* could not be deleted in Δ*ssrA* cells using the same procedure, suggesting that *CC1214* is essential in the absence of *trans*-translation.

Table 2-2 Doubling time of mutant strains

strain	doubling time
wt	103 ± 1
Δ*ssrA*	127 ± 1
CC1214::Ω	103 ± 1
CC1215::cat (sense)	101 ± 1
CC1215::cat (antisense)	99 ± 5
Δ*ssrA CC1215::cat* (sense)	122 ± 1
Δ*ssrA CC1215::cat* (antisense)	114 ± 4

A co-transduction experiment was used to confirm that loss of *CC1214* is lethal in the Δ*ssrA* background. A gene conferring kanamycin resistance was inserted in the chromosome of *C. crescentus CC1214::Ω* ~22 kb from the *CC1214* locus to make strain KCK428. Based on their separation on the chromosome, the kanamycin and spectinomycin resistance markers would be expected to co-transduce using phage ΦCR30 at a frequency of 45%. Consistent with this prediction, when a ΦCR30 lysate was prepared from KCK428 cells and used to infect wild-type *C. crescentus*, 52% of kanamycin-resistant transductants were also spectinomycin-resistant (Table 2-3). In contrast, when the ΦCR30 lysate was used to infect Δ*ssrA* cells, none of the 150 kanamycin-resistant colonies were spectinomycin-resistant. When Δ*ssrA* cells containing a plasmid-borne copy of *ssrA* were used as the recipient, the co-transduction frequency was

restored to 51%. Likewise, when Δ*ssrA* cells containing a plasmid-borne copy of *CC1214* were used as the recipient, the co-transduction frequency was 55%. These results indicate that *C. crescentus* cells lacking both *ssrA* and *CC1214* cannot grow under typical culture conditions.

Table 2-3: Co-transduction frequencies

recipient strain	%*CC1214::Ω* transductants	% *CC1214* transductants
wt	52	48
Δ*ssrA*	0	100
Δ*ssrA* p*ssrA*	51	49
Δ*ssrA* p*CC1214*	55	45
Δ*ssrA* p*CC1214*G30A	0	100

CC1215* interactions with *ssrA* are due to polar effects on *CC1214

The Tn-seq data indicated that *himar1* insertions in *CC1215* confer a competitive advantage in Δ*ssrA* cells. *CC1215* could be replaced with the *cat* gene, encoding chloramphenicol resistance, in both wild-type and Δ*ssrA* cells. Insertions of *cat* in the sense and antisense orientation relative to *CC1215* were recovered in both strains. Deletion of *CC1215* did not alter the growth rate of strains in liquid culture or on plates (Table 2-3), indicating that the increased recovery of *himar1* insertions in Δ*ssrA* cells in the Tn-seq experiment was not due to loss of the *CC1215* gene product. One likely explanation for the observed interaction between *CC1215* and *ssrA* is that *himar1* insertion resulted in increased expression of *CC1214*, which is immediately downstream of *CC1215*, making the Δ*ssrA* cells more fit. Consistent with that hypothesis, *CC1215* could not be replaced with an Ω cassette in Δ*ssrA* cells but in wild-type cells the *CC1215::Ω* mutation was viable. Because the Ω cassette contains transcriptional terminators flanking the antibiotic resistance gene, it produces strong polar effects (28).

***CC1214* hydrolyzes peptidyl-tRNA on nonstop translation complexes**

CC1214 was annotated as a peptidyl-tRNA hydrolase, but it has greater sequence similarity to *E. coli arfB*. Because purified *E. coli* ArfB can release peptidyl-tRNA from non-stop complexes in vitro, the CC1214 protein was purified and tested for non-stop ribosome release activity. In vitro transcription/translation assays were programmed with a DHFR gene lacking a stop codon (DHFR-ns) and resolved on bis-tris-polyacrylamide gels that preserve the peptidyl-tRNA bond. Ribosomes translating DHFR-ns reach the 3' end of the mRNA and cannot be released by RF1 or RF2, so the percentage of DHFR protein in the peptidyl-tRNA band is a measure of the ribosome release activity. For untreated reactions, 52% of the DHFR protein was in the peptidyl-tRNA band (Figure 2-2). When puromycin, a drug that hydrolyzes peptidyl-tRNA in the ribosome, was added to reactions prior to electrophoresis, 16% of the DHFR protein remained as peptidyl-tRNA. When purified CC1214 was added to reactions, 11% of the DHFR was in the peptidyl-tRNA, indicating that CC1214 can hydrolyze ~80% of peptidyl-tRNA from non-stop complexes under these conditions. This amount of release activity was similar to the release activity observed for RF1 and RF2 when the DHFR template contained a TAA stop codon (not shown).

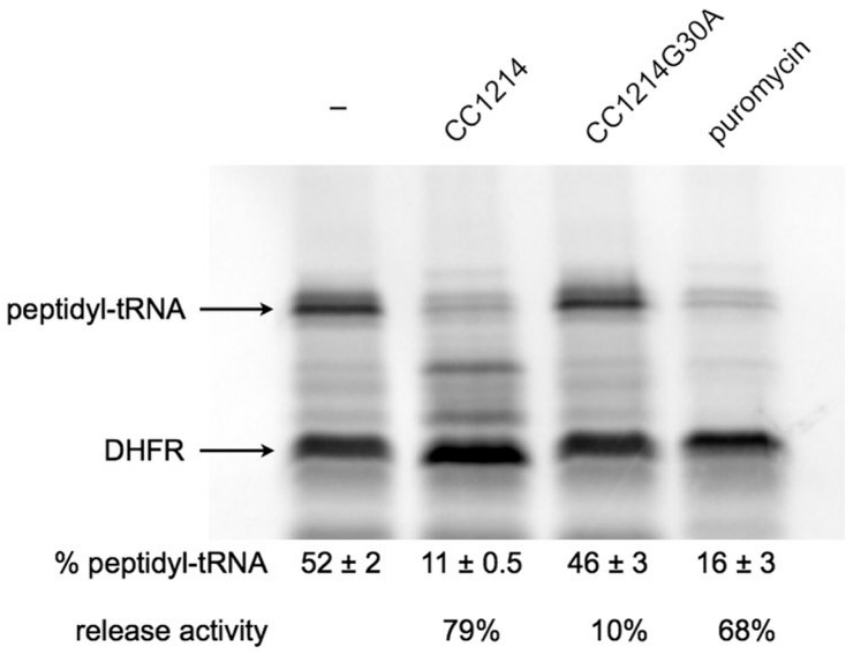

Figure **2-2**: CC1214 hydrolyzes peptidyl-tRNA on nonstop translation complexes. *In vitro* transcription/translation reactions were programmed with a DHFR gene lacking a stop codon, treated with CC1214, CC1214G30A, or puromycin, and resolved on a bis-Tris gel. The positions of free DHFR protein and DHFR peptidyl-tRNA are indicated. The average (± standard deviation) percentages of DHFR in the peptidyl-tRNA band from 3 experiments and the percent release activities with respect to the release activity in the untreated control are shown.

Bacterial factors that hydrolyze peptidyl-tRNA on the ribosome, including RF1, RF2, and ArfB, have a GGQ motif that is important for activity (10,29), but this motif is not found in Pth enzymes that hydrolyze free peptidyl-tRNA. A GGQ motif is conserved in CC1214. A variant of CC1214 in which the GGQ motif was replaced with GAQ (CC1214G30A) was purified and tested for non-stop ribosome release activity. In these reactions, 46% of the DHFR remained in the peptidyl-tRNA band, indicating that the GGQ motif is important for CC1214 activity. Consistent with that hypothesis, a plasmid-borne copy of *CC1214G30A* would not allow transduction of *CC1214::Ω* into Δ*ssrA* cells (Table 2-3).

Multicopy expression of CC1214 suppresses the Δ*ssrA* growth defect

C. crescentus lacking *ssrA* or *smpB* grow slowly (30). To determine if over-expression of CC1214 could suppress this phenotype, *CC1214* was cloned under control of a xylose-inducible promoter on a multicopy plasmid. During exponential growth phase in liquid culture, wild-type cells over-expressing CC1214 had a doubling time of 94 ± 3 min (Figure 2-3). Wild-type cells with an empty vector had a doubling time of 98 ± 5 min, indicating that over-expression of CC1214 does not have a large effect on growth rate when trans-translation is functional. Consistent with previously published data (30), the doubling time in ΔssrA cells with an empty vector was 125 ± 3 min. However, when CC1214 was over-expressed, the doubling time decreased to 108 ± 3 min. This partial suppression of the slow growth phenotype in ΔssrA cells suggests that release of non-stop complexes by CC1214 can compensate for the absence of trans-translation. Failure of CC1214 to fully restore the growth rate of Δ*ssrA* cells to that of wild-type cells suggests that additional functions of *trans*-translation, such as targeted proteolysis of the aberrant peptide, may also contribute to growth rate under these conditions.

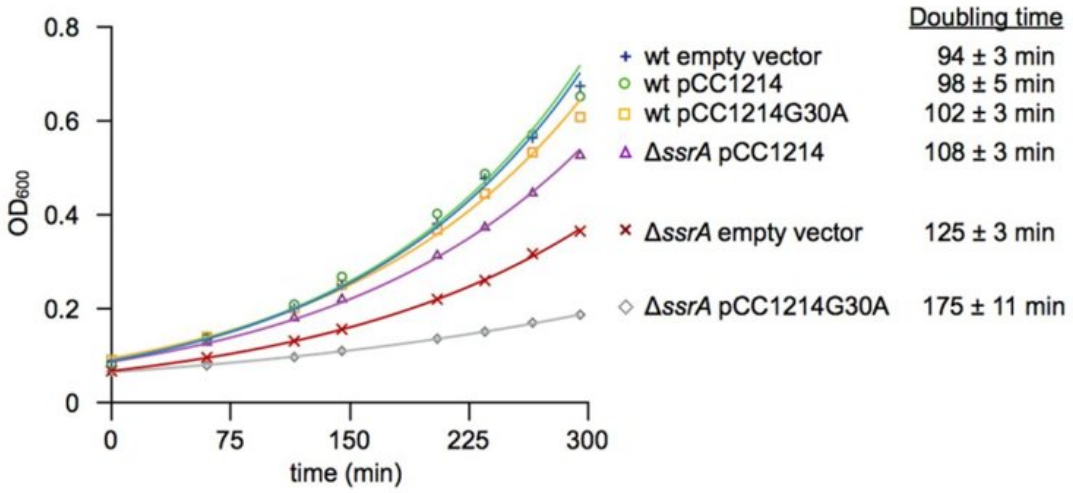

Figure 2-3: Overexpression of CC1214 suppresses the slow growth phenotype in *ssrA* cells. The growth of *C. crescentus* strains was monitored by measuring the optical density at 600 nm and plotted versus time. The doubling time (standard deviation) of each strain is indicated. wt, wild type.

Suppression by *CC1214* required non-stop ribosome release, because over-production of the inactive CC1214G30A variant resulted in a decrease in growth rate instead of an increase. In wild-type cells over-expressing *CC1214G30A* the doubling time was 102 ± 3 min, and in Δ*ssrA* cells over-expressing *CC1214G30A* the doubling time was 175 ± 11 min. These results suggest that the inactive mutant can compete with wild-type CC1214 for binding to non-stop ribosomes and inhibit non-stop ribosome release in the absence of *trans*-translation. Consistent with this interpretation, mutants of *E. coli* ArfB, RF1, and RF2 that alter the GGQ motif can bind non-stop ribosomes (26).

Genes encoding ArfB are widely distributed in bacteria

Because CC1214 has significant sequence similarity to the *E. coli* ArfB gene, including the catalytic GGQ motif and positively charged residues in the C-terminal tail that are required for release of non-stop ribosomes (Figure 2-4), and because CC1214 can release non-stop ribosomes in vitro and can support viability of strains deleted for *ssrA*, we propose that CC1214 and its homologs should be renamed ArfB. To determine how widely distributed genes encoding ArfB are within α-proteobacteria, 236 completed α-proteobacterial genomes were searched using BLAST (31) with the *C. crescentus* ArfB sequence. No homologs were identified in the Rickettsial branch (including *Rickettsia*, *Ehrlichia*, *Wolbachia*, *Candidatus* and *Anaplasma* species), but of the remaining 171 species, 80% encoded ArfB (Figure 2-5). To determine whether ArfB is abundant throughout other lineages of bacteria, BLAST searches were performed on the NCBI set of representative genomes for the 21 different bacterial phyla. Homologs were found in 11 phyla (Table 2-4). >60% of Planctomycetes, Chlorobi and β-proteobacteria species encoded homologs, and >40% of Cyanobacteria, Bacteroidetes, δ-Proteobacteria, Actinobacteria species had recognizable homologs.

```
Ccr       ---MPAMIEITSW-----LRIDE----DDLIWKATRASGPGGQHVNKTSTAIELRFDVRNSPYLPDDVKDRLTALAGSR
Eco       ------MIVISRH-----VAIPD----GELEITAIRAQGAGGQHVNKTSTAIHLRFDIRAS-SLPEYYKERLLAASHHL
Bja       ------MLRISRD-----LVIDE----DDIEIGFVRASGPGGQNVNKVSTSAQLRFDTRKL-TLPEDAVIRLARIAGQR
Mxa       MKTAMSISPTRRQAALDALKLDDESLLKACEVDYFIASGPGGQHRNTTASGVRLTHAPT--------------------
Sco       MEGMSGPHVIRGS-----VSLPE----AELVWRFSRSSGPGGQHVNTTDTAVELRFDLARTEALPEVWKQRALERLAGR
S6803     ------MLQITKS-----LSIAL----TEIEMTALRSQGAGGQNVNKVASAIHLRFDIGAS-SLPDVYKERLLNLNDQR
SL21      ----MSLIHLYRN-----LSIPD----SEIQFSASRASGKGGQHVNTTSSAVQLRFNIPGS-SLPEQVKSRLLAMSDSR
SNBC      ----MPNLKISNT-----VTLDE----NEIEISAIRAQGSGGQKVNKVSAAIHLRFDIAAS-SLPAFYKEKLLALKDKR
Bth       ---------MTLR-----YAFSP----DEVELTAVRAQGAGGQNVNKVSSAIHLRFDIRAS-SLPDDVKARLLARADQR
Dto       ------MLKINDN-----ISIPE----TQIQFQAIRAQGAGGQNVNKVSTAIHLRFDIQAS-SLPDQVKDKLLSLSDQR
                                :              :.* ***. *.. :. .* .

Ccr       MTQDGVLVLFAQGSRSQEMNRQDALDRLIDLIRRATE-KPKPRKATKPTYSSKLKRLETKSKRGTVKSGRGRV-KFDD
Eco       ISSDGVIVIKAQEYRSQELNREAALARLVAMIKELTT-EKKARRPTRPTRASKERRLASKAQKSSVKAMRGKV-R---
Bja       MTKDGVIVIHAQRFRTQERNRQDAIDRLTEILTEAMI-RPKPRRATRPTLGSKQRRLDGKKRRSDIKAGRGGG-RFDD
Mxa       -----ELSVSATERRSQVQNKGVALERLREGLKVLTF-VPKVRRATKPTAGSKRRRLEGKKRTSEKKALRNSKSGW--
Sco       L-SDGVVTVRASEHRSQWRNRETAAVRLAALLAEATAPPPRPRRPTRIPRGINERRLREKKQRSDTKRGRSAR-GWD-
S6803     ITKDGIVVIKAQSHRTQEQNREEALKRLQGLISSVIT-LPKPRRPTKPTRSSQRKRLDAKTKRSAVKALRKSI-DE--
SL21      LTTEGVLIIRSENERSQRRNRQDALERLREYFRQALK-PVKTRRPTRPGKAAKHRRLDSKKHRGRLKEGRKPP-PTPG
SNBC      ISKDGIVVIKSQQHRSQEQNREEALERLVELIKSVTV-TQKKRVPTKPTKGSVNRRLNSKKKHAGKKRLRGKV-ERE-
Bth       ITREGIVVIKAQEYRTQEKNRAAALARLDTLIRSAGV-TPRKRIATRPTRASKERRLAEKSRRSDVKSGRGRI-VE--
Dto       ISKTGVLVIKAQTFRTQEKNKEDALNRLQKIVQSVLV-KKKKRQKTRPKKGAVEKRLDSKTKNGRVKKLRKKV-DY--
                : : :   *:*  *:  *  **   .       : *  *:   .   :**  * : .  *  *
```

Figure **2-4**: ArfB homologs are found across the bacterial kingdom. ArfB sequences were aligned with Clustal Omega. Asterisks indicate conserved residues, colons indicate residues with strongly similar properties, and dots indicate residues with weakly similar properties. Residues which have been shown experimentally to be important for hydrolysis activity in *E. coli* are highlighted in green. Species abbreviations are as follows: Ccr, *Caulobacter crescentus*; Eco, *Escherichia coli*; Bja, *Bradyrhizobium japonicum*; Mxa, *Myxcoccus xanthus*; Sco, *Streptomyces coelicolor*; S6803, Synechocystis sp. strain PCC6803; SL21, *Spirochaeta* sp. strain L21-RPul-D2; SNBC, *Sulfurovum* sp. strain NBC37-1; Bth, *Burkholderia thailandensis*; and Dto, *Desulfobacula toluolica* strain Tol2.

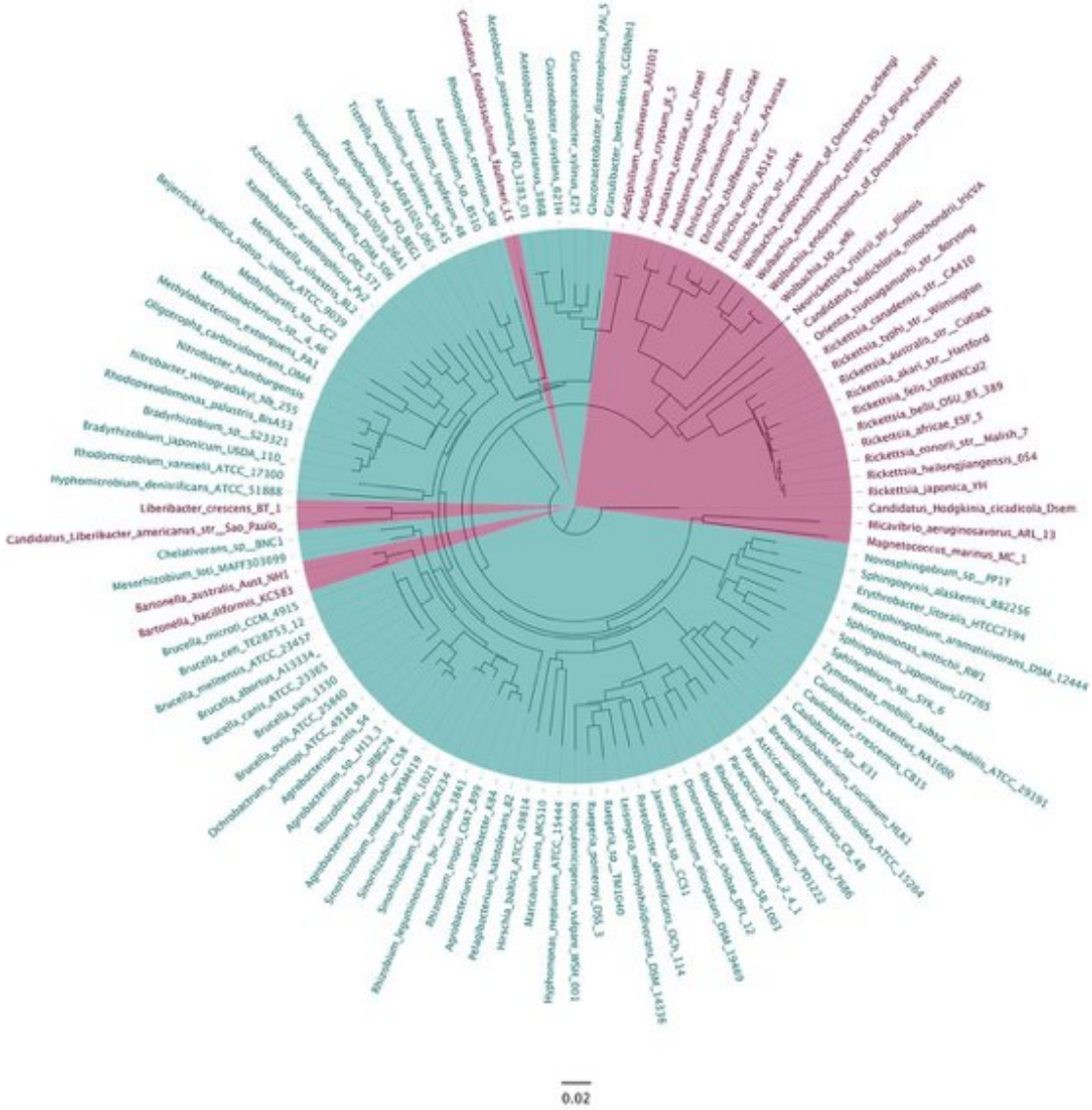

Figure **2-5**: ArfB homolog distribution in *Alphaproteobacteria*. A tree of alphaproteobacterial species generated using 16S rRNA gene sequences is shown, with species encoding ArfB homologs highlighted in turquoise and species that do not encode an ArfB homolog highlighted in magenta.

Table 2-4: Phylogenetic distribution of ArfB homologs

Phylum	Representative genomes	Number with ArfB	% with ArfB
Actinobacteria	174	83	48
Aquificae	9	0	0
Cyanobacteria	55	25	45
Chlorobi/Bacteroidetes	96	48	50
Firmicutes	268	0	0
α-Proteobacteria	178	102	57
β-Proteobacteria	102	75	74
ε- Proteobacteria	29	3	10
γ- Proteobacteria	271	101	37
δ- Proteobacteria	54	32	59
Fusobacteria	7	0	0
Spirochaetes	41	7	17
Chlamydiae/Verrucomicrobia	19	0	0
Planctomycetes	7	6	86
Tenericutes	48	0	0
Synergistetes	7	0	0
Thermotogae	17	0	0
Deinococcus	7	0	0
Chloroflexi	16	1	6
Armatimonadetes	1	0	0
Caldiserica	1	0	0
Chrysiogenetes	1	0	0
Deferribacteres	4	0	0
Dictyoglomi	2	0	0
Elusimicrobia	2	0	0
Fibrobacteres/Acidobacteria	9	0	0
Gemmatimonadetes	1	1	100
Nitrospirae	3	1	33
Thermodesulfobacteria	2	0	0
Total	1431	383	34

Materials and Methods

Bacterial Growth

Strains are described in Table 2-5. *C. crescentus* strains were grown at 30° C in PYE medium, supplemented with tetracycline (2 µg/ml), streptomycin (50 µg/ml), spectinomycin (100 µg/ml) or kanamycin (20 µg/ml) where appropriate. Culture growth was monitored by measuring the optical density at 600 nm.

Table 2-5: Strains and plasmids

Strain or Plasmid	Description	Source
CB15N	wild-type *C. crescentus*	(32)
CB15N Δ*ssrA*	In-frame deletion of *ssrA*	(30)
CB15N *CC1214::Ω*	*CC1214* replaced by omega cassette	This study
KCK 426	*E. coli* BL21 (DE3) pET28CC1214	This study
KCK 428	CB15N *CC1214::Ω* with Kan^R marker 22.4 kb downstream	This study
KCK429	CB15N *CC1215* replaced by *cat* gene in antisense orientation	This study
KCK430	CB15N *CC1215* replaced by *cat* gene in sense orientation	This study
KCK431	CB15N Δ*ssrA CC1215* replaced by *cat* gene in antisense orientation	This study
KCK432	CB15N Δ*ssrA CC1215* replaced by *cat* gene in sense orientation	This study
pML81	pJS14-derived plasmid with xylose promoter and NdeI site	M.T. Laub, unpublished
pML81-His6-CC1214	expresses CC1214 from xylose promoter	This study
pBBR1-tet	pBBR1 plasmid with *tetA* cloned into NotI site	gift from Camille Lochte, Pasteur

Strain or Plasmid	Description	Source
		Institute of Lille
pBBR-PBN	pBBR1-tet derived plasmid with PstI, BamHI, and NdeI cloning sites.	This study
pCC1214	expresses CC1214 from xylose promoter	This study
pACC1214G30A	expresses CC1214 active site mutant (GAQ) from xylose promoter	This study
pSsrA	expresses *ssrA* from its native promoter	(30)
pBGS18T(1349674–1350629)	pBGS18T containing targeting fragment 1349674–1350629	(33)
pET28CC1214	expresses CC1214 from T7 promoter	This study
pDHFR	expresses DHFR from T7 promoter	New England Biolabs
pMT425	plasmid with vanillate inducible promoter, *cat* gene	(34)

Plasmid construction

Oligonucleotide sequences are shown in Table 2-6. Plasmid pBBR-PBN was constructed by amplifying plasmid pBBR1-tet by PCR using primers BBRtetPBN1 and BBRtetPBN2, digesting the product with BamHI, and ligating to circularize the plasmid. Plasmid pCC1214 was constructed by amplifying the *CC1214* gene by PCR using primers 1214_His6F and 1214_Rev, digesting the resulting DNA with NdeI and HindIII, and ligating the product into pML81 cut with the same enzymes to create plasmid pML81-His6-1214. The xylose promoter and *CC1214* were then amplified from pML81-His6-1214 by PCR using primers PstXyl224F and 1214PstIR and ligated into plasmid pBBR-PBN cut with PstI, to produce pCC1214. pCC1214G30A was made

by primer extension mutagenisis. pML81-His6-1214 was used as template in two separate PCR reactions using primers PstXyl224F and G30A_R2 or primers G30A_F2 and 1214PstIR. The products of these reactions were used in a second PCR with primers PstXyl224F and 1214PstIR. The resulting product was cloned into the PstI site of pBN and sequenced to verify the mutation. pET28CC1214 was constructed by amplifying *CC1214* with primers 1214_Fwd and 1214_Rev, digesting with NdeI and HindIII, and ligating into pET28b that had also been digested with NdeI and HindIII.

Table 2-6: DNA oligonucleotides

Primer	Sequence
1214_His6F	GGTGATCATATGCATCATCATCATCATCACCCCGCGATGATCGAG
1214PstIR	TATCTGCAGTTAGTCGTCGAATTTCACCCGCC
PstXyl224F	GTCTCGGGCCGTCTCTTGGCTGCAGGTAGAAGGCGCCCTC
G30A_F2	GCCTCGGGTCCCGGCGCCCAGCACGTCAACAAGACCTCG
G30A_R2	CGAGGTCTTGTTGACGTGCTGGGCGCCGGGACCCGAGGC
1214_UF	ATTCCACTAGTCAGCGCCACCGGCTCCACCCG
1214_UR	TCGATGAATTCGGGCATGCGATCACCGGGGC
1214_DF	CGGGTGAATTCGACGACTAACGACGGCTCAAG
1214_DR	TGGACGCTAGCGAAGAACGGCAAGCGACGCTGAAGAA
1214_Fwd	GGTGATCATATGCCCGCGATGATCGAGAT
1214_Rev	GGGTGAAATTCGACGACTAAGCTTGGCTC
1215_DF	CGCGGAATTCCGGTGATCGCATGCCCGCGA
1215_DR	CGTGAAGGAAAAGCGGATCCAGCCCCGGC
1215_UF	CGCGGCTAGCTGGTCCGGCTGTTCGGC
1215_UR	TCGATGAATTCCGTGGCGACCATCGCGCGAGA
HAF_T7	CGAAATTAATACGACTCACTATAGGG
UTR_DHFR_FL	AAACCCCTCCGTTTAGAGAGGGGTTTTGCTAGTATCCGCCGCTCC AGAATCTCAAAGCAA
1214_ScrnF	CAAGACCTTTGTCGTCGCCAACAGCTGG
1214_ScrnR	ACCATGACCCTCTATCTGCAGAAGATGCTG

Primer	Sequence
1215_ScrnF	CTGGAAGCAATATATGCGCCGCCAGAC
1215_ScrnR	CACGTCGAAGCGCAGCTCGATG
BBRtetPBN1	CTGCAGGGATCCCCATGGCTTGATCGGCCTTCTTGC
BBRtetPBN1	CCATGGGGATCCCTGCAGACCTTCGGGAGCGCCTGA

Transposon mutagenesis and sequencing (Tn-Seq)

Overnight cultures of wild-type and Δ*ssrA C. crescentus* strains were grown and mutagenized with a *himar1* transposon. >100,000 colonies were pooled, and chromosomal DNA was extracted and sequenced using an Illumina HiSeq 2000 as previously described (35). Datasets were analyzed in SeqMonk V0.27.0 by normalizing to the total number of reads in the largest dataset, adding 1 to eliminate infinite and zero ratios, and calculating the ratio of insertions in Δ*ssrA* to wild-type cultures (35).

Genetic Deletions

Regions of ~1.5 kb flanking *CC1214* were amplified by PCR using primers 1214_UF and 1214_UR or 1214_DF and 1214_DR. The products were cut with SpeI and EcoRI, or NheI and EcoRI respectively, and ligated into the SpeI and NheI sites of integrating plasmid pNPTS138. The omega cassette from pHP45Ω was inserted at the EcoRI site between the flanking regions. The resulting plasmid was used to construct CB15N*CC1214::Ω* using the two-step recombination method (27). Mutants were selected on spectinomycin and the *CC1214::Ω* mutation was confirmed by PCR with primers 1214_ScrnF and 1214_ScrnR. CB15N*CC1215::cat* was constructed similarly, using primers 1215_UF and 1215_UR to amplify a region ~1.5 kb

upstream of 1215. A downstream flanking region was amplified using primers 1215_DF and 1215_DR. PCR fragments were digested with NheI and EcoRI or BamHI and EcoRI, and ligated into pNPTS138 digested with NheI and BamHI. The *cat* gene from pMT425 was amplified with primers cat220F and EcoCatR, cut with EcoRI, and ligated into the EcoRI site between the two flanking regions. The direction of the *cat* insertion was determined by PCR, and two-step recombination was used to construct CB15N*CC1215::cat* with the *cat* cassette facing in either the sense (KCK429) or antisense (KCK430) direction with respect to *CC1215*. The presence of the mutation in selected clones was verified by PCR using primers 1215_ScrnF and 1215_ScrnR. To construct KCK 431 and KCK 432, ΦCR30 phage lysate was prepared from KCK 429 and KCK 430, respectively, and used to infect CB15N Δ*ssrA*. The presence of the mutation was verified using primers 1215_ScrnF and 1215_ScrnR.

Phage transduction

A kanamycin-resistance marker was inserted 22.4 kb from *CC1214::Ω* to make strain KCK 428 by transforming CB15N *CC1214::Ω* with the nonreplicating plasmid pBGS18T(1349674–1350629) (33). ΦCR30 phage lysate was prepared from KCK 428 and used to infect CB15N or CB15N Δ*ssrA* (36). Transductants were selected on plates containing kanamycin plates and patched onto plates containing streptomycin and spectinomycin to determine the co-transduction frequency. The expected frequency was predicted by the equation log(distance)= –1.56(ctf) + 5.06 where distance represents the number of bases between the two makers, and ctf represents co-transduction frequency (33).

Purification of CC1214

KCK 426 was grown to $OD_{600} \approx 0.8$ and expression of CC1214 was induced by addition of IPTG to 1 mM. Cells were harvested by centrifugation, resuspended in guanidinium lysis buffer (6 M guanidine hydrochloride, 20 mM sodium phosphate [pH 7.8], 400 mM NaCl), and sonicated. The lysate was cleared by centrifugation at 11,000 x g for 10 min. A column was loaded with Ni-NTA agarose (Qiagen) that had been equilibrated with denaturing binding buffer (8 M urea, 20 mM sodium phosphate [pH 7.8], 500 mM NaCl). The column was loaded with cleared lysate, and washed by adding 10 vol denaturing binding buffer and rocking for 15 min. The column was then washed with 10 vol denaturing wash buffer (8 M urea, 20 mM sodium phosphate [pH 6.0] and 500 mM NaCl) and 10 vol denaturing wash buffer equilibrated to pH 5.3. Bound protein was eluted with denaturing elution buffer (8 M urea, 20 mM sodium phosphate [pH 4.0] and 500 mM NaCl) and visualized by SDS-PAGE. Fractions containing 6XHis-ArfB were dialyzed against Buffer H (10 mM HEPES [pH 7.5], 150 mM NaCl).

In vitro translation and peptidyl-tRNA hydrolysis assays

CC1214 was assayed for peptidyl hydrolysis activity using the PURExpress system (New England Biolabs) similar to previous assays (37)with some modifications. Non-stop DHFR was amplified using primers HAF_T7 and UTR_DHFR_FL and used as template for the reaction. After 1 h incubation at 37 °C, CC1214 or CC1214G30A was added to a final concentration of 200 nM. A control sample was treated with 70 µg/ml puromycin. After 1 h incubation at 37 °C, total protein was precipitated by addition of acetone, resuspended in sample loading buffer (5 mM sodium bisulfite, 50 mM MOPS, 50 mM tris base, 1 µM EDTA, 0.1% SDS, 5% glycerol, 0.01% xylene cyanol, 0.01% bromophenol blue), and resolved on a bis-tris gel using MOPS

running buffer.

Sequence homology searches

The protein sequence of CC1214 was used in a tblastn search (31)of the June 2014 NCBI database of representative microbial genomes. An e-value cutoff of 10^{-7} and a molecular weight cutoff of 20 kDa were used to identify homologs. In the case where a homolog was identified for a given phylum, that homolog was then used in a tblastn search of the remaining representative genomes in that phylum. If a homolog was not found, tblastn searches were performed with homologs from closely related phyla. A tree of α-proteobacteria was constructed by collecting 16S rDNA sequences from PATRIC (38)and aligning them with BLAST. The tree was viewed and colored using Figtree software (version v1.4.2 [http://tree.bio.ed.ac.uk/software/figtree/]).

Discussion

The results described here show that *C. crescentus* can survive without *trans*-translation only because they have ArfB to release non-stop translation complexes. ArfB can hydrolyze peptidyl-tRNA on non-stop translation complexes in vitro, and *C. crescentus* cells require either *trans*-translation or ArfB activity for viability. Although *E. coli* ArfB was identified in a multicopy suppressor screen as a gene that would allow both *ssrA* and *arfA* to be deleted, *E. coli* ArfB will not support viability as the only non-stop complex release activity from its chromosomal locus (26). In contrast, the *C. crescentus* ArfB in its wild-type locus can act as a backup system for *trans*-translation during culture growth. *E. coli* and *C. crescentus* ArfB have similar release activity *in vitro*, so it is likely that *E. coli* ArfB does not act as a backup system for *trans*-translation solely because it is expressed at low levels.

Homology searches using the *C. crescentus* ArfB sequence revealed that several other species that do not require *trans*-translation encode ArfB, including *Bradyrhizobium japonicum* and *Streptomyces coelicolor* (39,40). With the addition of ArfB homologs similar to the *C. crescentus* protein, all species in which *ssrA* or *smpB* have been deleted encode either ArfB or ArfA, with the exception of *Francisella tularensis* and *Bacillus subtilis.* Tn-seq experiments or other synthetic-lethal screens should reveal whether *F. tularensis* and *B. subtilis* have a different backup system for *trans*-translation. The essentiality of nonstop ribosome rescue in close relatives of these species suggests that resolution of nonstop ribosomes is also essential in these species. A screen for this alternative rescue pathway in *B. subtilis* and potential candidates are described in the appendix of this dissertation.

C. crescentus cells lacking *trans*-translation grow slowly and have a cell cycle defect, and this phenotype is not complemented by *ssrA-DD*, a variant that releases non-stop complexes but does not target the nascent polypeptide for rapid degradation (30). These results were interpreted

to indicate that degradation of one or more tagged proteins was required for normal growth in *C. crescentus*. However, this hypothesis is not consistent with the observation that over-expression of *arfB* in Δ*ssrA* cells restored the wild-type growth rate. Instead, it appears that the slow growth phenotype in cells lacking *trans*-translation is caused in part by failure to release non-stop translation complexes, similar to phenotypes observed for *ssrA* deletions in other species. The inability of ArfB over-expression to fully restore the Δ*ssrA* growth rate suggests that *trans*-translation may resolve nonstop ribosomes more rapidly than ArfB, or that targeting of aberrant peptides for proteolysis by *trans*-translation imparts an additional growth advantage.

Does *C. crescentus* ArfB perform unique functions for the cell, or is its only role to fill in when *trans*-translation activity is overwhelmed? This question is particularly interesting in *C. crescentus*, because the levels of tmRNA and SmpB fluctuate as a function of the cell cycle, with both molecules dropping to very low levels in early S phase (30,41). Nevertheless, ArfB does not play a critical role in the cell cycle, because strains deleted for *arfB* grow at the same rate as wild type. Moreover, RNA-seq and ribosome profiling experiments indicated that *arfB* is not highly transcribed or translated during growth in culture (28), and experiments on synchronized cultures indicated that *arfB* transcription is not cell-cycle regulated (42). Any role for ArfB other than acting as an emergency backup for *trans*-translation remains to be identified.

Mitochondria contain a protein, ICT1, that has 33% sequence identity with *C. crescentus* ArfB, and ICT1 has been shown to hydrolyze peptidyl-tRNA on non-stop translation complexes as well as at stop codons (43,44). ICT1 is clearly important for mitochondria, because it is conserved in all mitochondrial genomes and is essential in HeLa cells (28,43). Mitochondria are thought to derive from α-proteobacteria, likely from the Rickettsial lineage (45,46), and the distribution of tmRNA and ArfB in current α-proteobacteria suggests that early α-proteobacteria had both *trans*-translation and ArfB. In fact, mitochondria from some single-celled eukaryotes retain tmRNA and SmpB in addition to ArfB (8). Why did the Rickettsial lineage keep *trans*-

translation and lose ArfB, while mitochondria in higher eukaryotes kept the ArfB homolog and lost *trans*-translation? Presumably, bacteria retain *trans*-translation because it targets the nascent polypeptide and non-stop mRNA for degradation in addition to releasing the ribosome. Mitochondria contain homologs of bacterial ClpXP, the protease that degrades most tmRNA-tagged proteins, so proteolysis of tagged proteins should have occurred in early mitochondria. It is possible that mitochondria have a different pathway to remove incomplete proteins, limiting the advantage of *trans*-translation. Alternatively, the simplicity of the single-chain ArfB might have been advantageous during genome reduction.

References

1. Laursen BS, Sørensen HP, Mortensen KK, Sperling-Petersen HU. Initiation of protein synthesis in bacteria. Microbiology and Molecular Biology Reviews. American Society for Microbiology; 2005 Mar;69(1):101–23.

2. Schmeing TM, Ramakrishnan V. What recent ribosome structures have revealed about the mechanism of translation. Nature. Nature Publishing Group; 2009 Oct 29;461(7268):1234–42.

3. Keiler KC, Waller PR, Sauer RT. Role of a peptide tagging system in degradation of proteins synthesized from damaged messenger RNA. Science. 1996 Feb 16;271(5251):990–3.

4. Keiler KC, Ramadoss NS. Bifunctional transfer-messenger RNA. Biochimie. 2011 Nov;93(11):1993–7.

5. Keiler KC. Biology of trans-translation. Annu Rev Microbiol. Annual Reviews; 2008;62(1):133–51.

6. Keiler KC. Mechanisms of ribosome rescue in bacteria. Nat Rev Microbiol. Nature Publishing Group; 2015 May;13(5):285–97.

7. Moore SD, Sauer RT. The tmRNA system for translational surveillance and ribosome rescue. Annu Rev Biochem. Annual Reviews; 2007;76(1):101–24.

8. Hudson CM, Lau BY, Williams KP. Ends of the line for tmRNA-SmpB. Front Microbiol. Frontiers; 2014;5(93):421.

9. Keiler KC, Feaga HA. Resolving nonstop translation complexes is a matter of life or death. Journal of Bacteriology. American Society for Microbiology; 2014 Jun;196(12):2123–30.

10. Chadani Y, Ono K, Ozawa S-I, Takahashi Y, Takai K, Nanamiya H, et al. Ribosome rescue by Escherichia coli ArfA (YhdL) in the absence of trans-translation system. Molecular Microbiology. Blackwell Publishing Ltd; 2010 Nov;78(4):796–808.

11. Chadani Y, Ito K, Kutsukake K, Abo T. ArfA recruits release factor 2 to rescue stalled ribosomes by peptidyl-tRNA hydrolysis in Escherichia coli. Molecular Microbiology. Blackwell Publishing Ltd; 2012 Oct;86(1):37–50.

12. Shimizu Y. ArfA recruits RF2 into stalled ribosomes. J Mol Biol. 2012 Nov 2;423(4):624–31.

13. Schaub RE, Poole SJ, Garza-Sánchez F, Benbow S, Hayes CS. Proteobacterial ArfA peptides are synthesized from non-stop messenger RNAs. J Biol Chem. American Society for Biochemistry and Molecular Biology; 2012 Aug 24;287(35):29765–75.

14. Garza-Sánchez F, Schaub RE, Janssen BD, Hayes CS. tmRNA regulates synthesis of the ArfA ribosome rescue factor. Molecular Microbiology. Blackwell Publishing Ltd; 2011 Jun;80(5):1204–19.

15. Ramadoss NS, Zhou X, Keiler KC. tmRNA is essential in Shigella flexneri. Herman C, editor. PLoS ONE. Public Library of Science; 2013;8(2):e57537.

16. van Opijnen T, Bodi KL, Camilli A. Tn-seq: high-throughput parallel sequencing for fitness and genetic interaction studies in microorganisms. Nat Methods. Nature Publishing Group; 2009 Oct;6(10):767–72.

17. Goodman AL, McNulty NP, Zhao Y, Leip D, Mitra RD, Lozupone CA, et al. Identifying genetic determinants needed to establish a human gut symbiont in its habitat. Cell Host Microbe. 2009 Sep 17;6(3):279–89.

18. Langridge GC, Phan M-D, Turner DJ, Perkins TT, Parts L, Haase J, et al. Simultaneous assay of every Salmonella Typhi gene using one million transposon mutants. Genome Res. Cold Spring Harbor Lab; 2009 Dec;19(12):2308–16.

19. Gawronski JD, Wong SMS, Giannoukos G, Ward DV, Akerley BJ. Tracking insertion mutants within libraries by deep sequencing and a genome-wide screen for Haemophilus genes required in the lung. Proc Natl Acad Sci USA. National Acad Sciences; 2009 Sep 22;106(38):16422–7.

20. van Opijnen T, Camilli A. Transposon insertion sequencing: a new tool for systems-level analysis of microorganisms. Nat Rev Microbiol. Nature Publishing Group; 2013 Jul;11(7):435–42.

21. Schaub RE, Hayes CS. Deletion of the RluD pseudouridine synthase promotes SsrA peptide tagging of ribosomal protein S7. Molecular Microbiology. Blackwell Publishing Ltd; 2011 Jan;79(2):331–41.

22. Shoji S, Janssen BD, Hayes CS, Fredrick K. Translation factor LepA contributes to tellurite resistance in Escherichia coli but plays no apparent role in the fidelity of protein synthesis. Biochimie. 2010 Feb;92(2):157–63.

23. Ude S, Lassak J, Starosta AL, Kraxenberger T, Wilson DN, Jung K. Translation elongation factor EF-P alleviates ribosome stalling at polyproline stretches. Science. American Association for the Advancement of Science; 2013 Jan 4;339(6115):82–5.

24. Singh NS, Varshney U. A physiological connection between tmRNA and peptidyl-tRNA hydrolase functions in Escherichia coli. Nucleic Acids Research. Oxford University Press; 2004;32(20):6028–37.

25. Hayes CS, Bose B, Sauer RT. Stop codons preceded by rare arginine codons are efficient determinants of SsrA tagging in Escherichia coli. Proc Natl Acad Sci USA. National Acad

Sciences; 2002 Mar 19;99(6):3440–5.

26. Chadani Y, Ono K, Kutsukake K, Abo T. Escherichia coli YaeJ protein mediates a novel ribosome-rescue pathway distinct from SsrA- and ArfA-mediated pathways. Molecular Microbiology. Blackwell Publishing Ltd; 2011 May;80(3):772–85.

27. Gay P, Le Coq D, Steinmetz M, Berkelman T, Kado CI. Positive selection procedure for entrapment of insertion sequence elements in gram-negative bacteria. Journal of Bacteriology. American Society for Microbiology (ASM); 1985 Nov;164(2):918–21.

28. Schrader JM, Zhou B, Li G-W, Lasker K, Childers WS, Williams B, et al. The coding and noncoding architecture of the Caulobacter crescentus genome. Hayes CS, editor. PLoS Genet. Public Library of Science; 2014 Jul;10(7):e1004463.

29. Mora L, Heurgué-Hamard V, Champ S, Ehrenberg M, Kisselev LL, Buckingham RH. The essential role of the invariant GGQ motif in the function and stability in vivo of bacterial release factors RF1 and RF2. Molecular Microbiology. 2003 Jan;47(1):267–75.

30. Keiler KC, Shapiro L. TmRNA is required for correct timing of DNA replication in Caulobacter crescentus. Journal of Bacteriology. American Society for Microbiology (ASM); 2003 Jan;185(2):573–80.

31. Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ. Basic local alignment search tool. J Mol Biol. Academic Press; 1990 Oct 5;215(3):403–10.

32. Evinger M, Agabian N. Envelope-associated nucleoid from Caulobacter crescentus stalked and swarmer cells. Journal of Bacteriology. American Society for Microbiology (ASM); 1977 Oct;132(1):294–301.

33. West L, Yang D, Stephens C. Use of the Caulobacter crescentus genome sequence to develop a method for systematic genetic mapping. Journal of Bacteriology. American Society for Microbiology (ASM); 2002 Apr;184(8):2155–66.

34. Thanbichler M, Iniesta AA, Shapiro L. A comprehensive set of plasmids for vanillate- and xylose-inducible gene expression in Caulobacter crescentus. Nucleic Acids Research. Oxford University Press; 2007;35(20):e137–7.

35. Murray SM, Panis G, Fumeaux C, Viollier PH, Howard M. Computational and genetic reduction of a cell cycle to its simplest, primordial components. Lichten M, editor. PLoS Biol. Public Library of Science; 2013 Dec;11(12):e1001749.

36. Ely B, Johnson RC. Generalized Transduction in CAULOBACTER CRESCENTUS. Genetics. Genetics Society of America; 1977 Nov;87(3):391–9.

37. Ramadoss NS, Alumasa JN, Cheng L, Wang Y, Li S, Chambers BS, et al. Small molecule inhibitors of trans-translation have broad-spectrum antibiotic activity. Proc Natl Acad Sci USA. National Acad Sciences; 2013 Jun 18;110(25):10282–7.

38. Wattam AR, Abraham D, Dalay O, Disz TL, Driscoll T, Gabbard JL, et al. PATRIC, the bacterial bioinformatics database and analysis resource. Nucleic Acids Research. Oxford

University Press; 2014 Jan;42(Database issue):D581–91.

39. Ebeling S, Kündig C, Hennecke H. Discovery of a rhizobial RNA that is essential for symbiotic root nodule development. Journal of Bacteriology. American Society for Microbiology (ASM); 1991 Oct;173(20):6373–82.

40. Yang C, Glover JR. The SmpB-tmRNA tagging system plays important roles in Streptomyces coelicolor growth and development. Herman C, editor. PLoS ONE. Public Library of Science; 2009;4(2):e4459.

41. Hong S-J, Tran Q-A, Keiler KC. Cell cycle-regulated degradation of tmRNA is controlled by RNase R and SmpB. Molecular Microbiology. Blackwell Science Ltd; 2005 Jul;57(2):565–75.

42. Fang G, Passalacqua KD, Hocking J, Llopis PM, Gerstein M, Bergman NH, et al. Transcriptomic and phylogenetic analysis of a bacterial cell cycle reveals strong associations between gene co-expression and evolution. BMC Genomics. BioMed Central; 2013;14(1):450.

43. Richter R, Rorbach J, Pajak A, Smith PM, Wessels HJ, Huynen MA, et al. A functional peptidyl-tRNA hydrolase, ICT1, has been recruited into the human mitochondrial ribosome. EMBO J. 2010 Feb 25;29(6):1116–25.

44. Akabane S, Ueda T, Nierhaus KH, Takeuchi N. Ribosome Rescue and Translation Termination at Non-Standard Stop Codons by ICT1 in Mammalian Mitochondria. Larsson N-G, editor. PLoS Genet. 2014 Sep 18;10(9):e1004616–14.

45. Williams KP, Sobral BW, Dickerman AW. A robust species tree for the alphaproteobacteria. Journal of Bacteriology. American Society for Microbiology; 2007 Jul;189(13):4578–86.

46. Pittis AA, Gabaldón T. Late acquisition of mitochondria by a host with chimaeric prokaryotic ancestry. Nature. Nature Publishing Group; 2016 Mar 3;531(7592):101–4.

Chapter 3

Human cells require nonstop ribosome rescue activity in mitochondria

Adapted from:

Feaga HA, Quickel MD, Hankey-Giblin PA, Keiler KC. 2016. Human cells require nonstop ribosome rescue activity in mitochondria. *PLOS Genetics*. 12(3):e1005964.

Special thanks to Mike Quickel of the Hankey-Giblin lab for the experiment in Figure 5 showing rescue of ICT1 silencing by ArfB.

Abstract

Bacteria use *trans*-translation and the alternative rescue factors ArfA and ArfB to hydrolyze peptidyl-tRNA on ribosomes that stall near the 3' end of an mRNA during protein synthesis. The eukaryotic protein ICT1 is homologous to ArfB. In vitro ribosome rescue assays of human ICT1 and *Caulobacter crescentus* ArfB showed that these proteins have the same activity and substrate specificity. Both ArfB and ICT1 hydrolyze peptidyl-tRNA on nonstop ribosomes or ribosomes stalled with ≤6 nucleotides extending past the A site, but are unable to hydrolyze peptidyl-tRNA when the mRNA extends ≥14 nucleotides past the A site. ICT1 provided sufficient ribosome rescue activity to support viability in *C. crescentus* cells that lacked both *trans*-translation and ArfB. Likewise, expression of ArfB protected human cells from death when ICT1 was silenced with siRNA. These data indicate that ArfB and ICT1 are functionally interchangeable, and demonstrate that ICT1 is a ribosome rescue factor. Because ICT1 is essential in human cells, these results suggest that ribosome rescue activity in mitochondria is required in humans.

Introduction

The presence of a stop codon at the end of an open reading frame signals that the nascent protein is complete. Decoding of the stop codon by a release factor results in peptidyl-tRNA hydrolysis, releasing the completed protein and allowing the ribosome to be recycled (1). Specific contacts between the release factors and bases in the stop codon are required for efficient catalysis of peptidyl-tRNA hydrolysis (2). This stop codon recognition is necessary to prevent release factors from acting at sense codons and prematurely terminating translation. However, ribosomes can sometimes translate to the end of an mRNA without terminating at an in-frame stop codon. Translation cannot terminate normally at these "nonstop" complexes, because there is no stop codon in the decoding center to promote release factor activity. Ribosomes must be rescued from nonstop complexes so they can be recycled for productive protein synthesis (3,4).

In bacteria, nonstop complexes are rescued primarily by *trans*-translation. During *trans*-translation, a small protein, SmpB and a specialized RNA, tmRNA, recognize a nonstop complex and release the ribosome at a stop codon within tmRNA. *trans*-Translation also targets the nascent polypeptide and mRNA from the nonstop complex for degradation (3,5). Genes encoding tmRNA or SmpB have been identified in >99.9% of sequenced bacterial genomes (4). *trans*-Translation is essential in some bacteria (6-8), but other species can survive without *ssrA* (encoding tmRNA) and *smpB* (9-11). Some species, such as *C. crescentus*, have a severe growth defect when *ssrA* is deleted (12). In other species, such as *Escherichia coli*, there is a relatively mild phenotype (13). Synthetic-lethal screens have identified two alternative rescue factors, ArfA and ArfB, that can rescue nonstop complexes in the absence of *trans*-translation (14-16). ArfA, found in *E. coli* and closely related bacteria, allows the release factor RF-2 to hydrolyze peptidyl-tRNA on nonstop ribosomes (17-20). ArfB, found in *C. crescentus* and species from many phyla, contains a catalytic domain similar to release factors but does not include domains required for

stop codon recognition. ArfB catalyzes hydrolysis of peptidyl-tRNA on nonstop ribosomes (15,16,21). The C-terminal tail of ArfB is important for its activity (22), and structural studies suggest that it binds in the empty mRNA channel of nonstop ribosomes, similar to the C-terminal tail of SmpB (23). *arfA* is essential in *E. coli* Δ*ssrA* cells (14), and *arfB* is essential in *C. crescentus* Δ*ssrA* cells (21), indicating that these species require at least one ribosome rescue mechanism. These observations have led to the suggestion that ribosome rescue activity may be essential for most or all bacteria (5).

Eukaryotes use Dom34/Pelota and Hbs1 to rescue ribosomes from nonstop mRNAs during translation in the cytoplasm (24,25), but factors required for this system are not present in mitochondria. Mammals have an ArfB homolog, ICT1, which is encoded in the nucleus and transported to mitochondria (26). Knockdown experiments have demonstrated that ICT1 is essential in human cells (26,27). Knockdown of ICT1 results in a decrease in protein synthesis in mitochondria (26), increased reactive oxygen species production, and apoptotic cell death (27).

Conflicting models have been proposed to explain why ICT1 is essential (28-30). Like ArfB, ICT1 can hydrolyze peptidyl-tRNA on *E. coli* nonstop ribosomes in vitro (22). Because ICT1 can also hydrolyze peptidyl-tRNA on *E. coli* ribosomes assembled on short mRNAs with a stop or sense codon in the A site, it has been proposed to act as a general release factor that can terminate translation at any codon (26). ICT1 has also been proposed to act on ribosomes stalled in the middle of an mRNA based on its ability to promote protein synthesis in reactions stalled by omission of a cognate release factor or tRNA (31) . Two mRNAs encoded in human mitochondria terminate with an AGA or AGG codon, so if ICT1 can act as non-specific release factor it might terminate translation of these messages. However, the sequence similarity between ICT1 and ArfB suggests that these factors are likely to have the same activity. ICT1 and ArfB share the conserved GGQ motif found in release factor catalytic domains, as well as residues in the C-terminal tail that are required for ArfB activity on nonstop ribosomes (Figure 3-1) (22,31)

```
H.sapiens ICT1      MAATRCLRWGLSRAGVWLLPPPARCPRRALHKQKDGTEFKSIYSLDKLYPESQGSDTAWR
C.crescentus ArfB   ------------------------------------------------------------
E.coli ArfB         ------------------------------------------------------------

H.sapiens ICT1      VPNG-AKQADSDIPLDRLTISYCRSSGPGGQNVNKVNSKAEVRFHLATAEWIAEPVRQKI
C.crescentus ArfB   MPAMIEITSWLRIDEDDLIWKATRASGPGGQHVNKTSTAIELRFDVRNSPYLPDDVKDRL
E.coli ArfB         ---MIVISRHVAIPDGELEITAIRAQGAGGQHVNKTSTAIHLRFDIRASS-LPEYYKERL

H.sapiens ICT1      AITHKNKINRLGELILTSESSRYQFRNLADCLQKIRDMITEASQTPKEP--TKEDVKLHR
C.crescentus ArfB   TALAGSRMTQDGVLVLFAQGSRSQEMNRQDALDRLIDLIRRATEKPKPRKATKPTYSSKL
E.coli ArfB         LAASHHLISSDGVIVIKAQEYRSQELNREAALARLVAMIKELTTEKKARRPTRPTRASKE

H.sapiens ICT1      IRIENMNRERLRQKRIHSAVKTSR-RVDMD--
C.crescentus ArfB   K--------RLETKSKRGTVKSGRGRVKFDD-
E.coli ArfB         R--------RLASKAQKSSVKAMRGKVRSGRE
```

Figure **3-1**: ICT1 and ArfB share conserved residues that are required for release activity. Clustal Omega alignment of human ICT1 and ArfB proteins from *E. coli* and *C. crescentus.* Blue stars indicate residues required for ≥ 60% ICT1 peptidyl-tRNA hydrolysis activity on nonstop ribosomes (22). Red stars indicate residues required for ≥ 60% *E. coli* ArfB hydrolysis activity on nonstop ribosomes (22). The N-terminal extension of ICT1 contains the mitochondrial localization signal. The remaining 143 C-terminal residues, thought to constitute the active portion of ICT1, share 26% sequence identity with *C. crescentus* ArfB.

Using a direct assay for peptidyl-tRNA hydrolysis in vitro, we find that the substrate specificity of ICT1 is almost identical to that of ArfB. Both factors catalyze peptidyl-tRNA hydrolysis on ribosomes stalled with no mRNA in the A site, or with mRNA extending a short distance past the A site, but have little activity on ribosomes stalled in the middle of an intact mRNA. In addition, we find that ArfB and ICT1 are interchangeable in vivo, both in *C. crescentus* and in human cells. These data indicate that ICT1 is a ribosome rescue factor and cannot terminate translation in the middle of mRNA, and suggest that mitochondrial ribosome rescue activity is essential in humans.

Results

Human ICT1 hydrolyzes peptidyl-tRNA on nonstop translation complexes

To evaluate the substrate specificity of ICT1, we used a gel-based assay to measure peptidyl-tRNA hydrolysis on *E. coli* ribosomes translating protein from mRNA. In these experiments, protein is produced using in vitro transcription-translation reactions and the components are separated on Bis-Tris gels that preserve the ester bond in peptidyl-tRNA. The fraction of protein that remains in the peptidyl-tRNA band indicates the extent of peptidyl-tRNA hydrolysis during the reaction (21).

To confirm that ICT1 can hydrolyze peptidyl-tRNA on nonstop ribosomes in a manner similar to ArfB, a coupled transcription-translation system lacking RF1, RF2, and RF3 was used to express a *folA* gene (encoding DHFR) that lacked a stop codon. Consistent with previous observations of peptidyl-tRNA hydrolysis on nonstop ribosomes (15,16,21), addition of a release factor mixture containing RF-1, RF-2, and RF-3 to the reaction had little effect on the percentage of DHFR found in peptidyl-tRNA, but when ArfB was included 74 ± 1% of the DHFR was released (Figure 3-2). When ICT1 was included in the reaction, 78 ± 8% DHFR was released, indicating that ICT1 has a similar activity to ArfB on nonstop ribosomes.

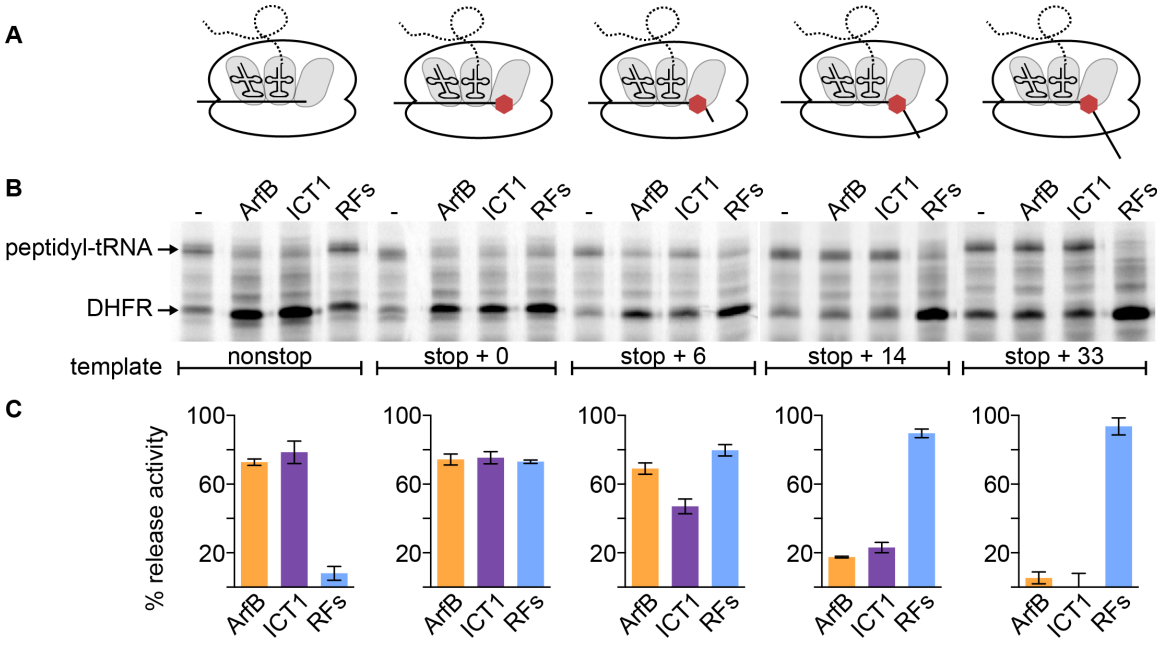

Figure **3-2**: ICT1 and ArfB hydrolyze peptidyl-tRNA on ribosomes near the 3' end of an mRNA. In vitro transcription/translation reactions were performed with template lacking a stop codon, or with template with 0, 6, 14, or 33 bases past the stop codon. (A) Cartoons depicting the expected result of translation in the absence of added rescue or release factors. (B) Representative autoradiograms of reactions resolved on Bis-Tris gels. (C) Column graphs show average release activity from ≥3 replicates with error bars indicating the standard deviation.

To determine if ICT1 and ArfB can also release ribosomes stalled with mRNA extending into or past the A site, the peptidyl-tRNA hydrolysis assay was repeated with longer *folA* templates. A stop codon was added to the end of the nonstop template, and 0, 6, 14, or 33 nucleotides were added after the stop codon. Each template was designed to produce an mRNA with a stem-loop at the 3' end to limit exonuclease activity during the reaction. Translation of these mRNAs will result in a ribosome stalled at the stop codon with peptidyl-tRNA in the P site. As expected, addition of release factors to reactions with any of these templates resulted in release of most of the peptidyl-tRNA (Figure 3-2). ICT1 and ArfB hydrolyzed peptidyl-tRNA as efficiently as release factors when the stop + 0 template was used. Substantial peptidyl-tRNA hydrolysis

activity by ICT and ArfB was also observed with the stop + 6 template, but significantly less activity was observed when the template had a longer sequence past the stop codon ($p < 0.001$). Almost no hydrolysis was observed with ICT1 or ArfB on the stop + 33 template. These results indicate that ICT1 and ArfB can hydrolyze peptidyl-tRNA on ribosomes stalled near the 3' end of an mRNA, and that a codon in the A site does not interfere with ribosome rescue. However, mRNAs that extend ≥ 14 bases past the A site substantially decrease activity of both ICT1 and ArfB.

Human ICT1 has ArfB activity in *C. crescentus*

The activity of ICT1 and ArfB in vitro was similar enough to suggest that ICT1 might substitute for ArfB in *C. crescentus.* In a previous study, we used genetic linkage analysis to demonstrate that deletions of *ssrA* and *arfB* are synthetically lethal in *C. crescentus* (21). A phage ΦCR30 lysate was prepared from strain KCK 428. In this strain, *arfB* is replaced with a copy of the *aadA* gene (conferring resistance to spectinomycin) and a copy of the *nptII* gene (conferring resistance to kanamycin) is inserted in the chromosome 22 kb from the *arfB* locus. The co-transduction frequency of the two antibiotic resistance markers could then be measured by infecting recipient cells with phage, selecting for transductants on kanamycin, and screening the kanamycin-resistant cells for spectinomycin resistance. Based on their relative locations on the chromosome, the predicted frequency with which the *aadA* and *nptII* genes should co-transduce when *arfB* is not essential is 45% (32). Consistent with this prediction, when lysate is used to infect wild-type cells, *arfB::aadA* is recovered at a frequency of 51 ± 8 % When this lysate was used to infect Δ*ssrA* cells, transductants with the *arfB* deletion were not recovered, indicating that *arfB* is essential in the absence of *ssrA*. To determine if ICT1 could provide ribosome rescue

activity to support viability of *C. crescentus*, the co-transduction experiment was repeated using Δ*ssrA* cells that contained ICT1 (Figure 3-3). When the Δ*ssrA* strain expressed ICT1 from a plasmid, *arfB* could be deleted, as indicated by the co-transduction of *arfB::aadA* with the *nptII* gene. The co-transduction frequency of *aadA* and *nptII* into Δ*ssrA* cells expressing ArfB or ICT1 was not significantly different (p = 0.12), indicating that ICT1 can suppress the synthetic-lethal phenotype of deleting *arfB* and *ssrA*. In contrast, when Δ*ssrA* cells harboring empty plasmid were used as the recipient, none of >500 kanamycin-resistant transductions were resistant to spectinomycin (Figure 3-3).

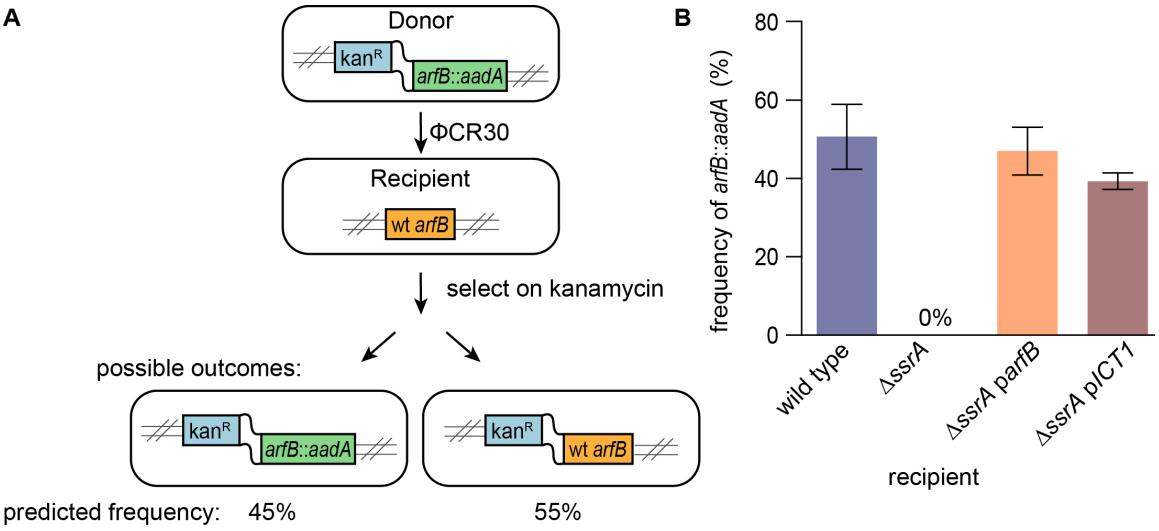

Figure **3-3**: ICT1 ribosome release activity supports viability in C. crescentus. A co-transduction experiment was used to test whether ICT1 complements the synthetic lethal phenotype of deleting arfB and ssrA. (A) Cartoon depicting the co-transduction experiment and predicted frequency of the outcomes if arfB were not essential. (B) Column graph indicating the average co-transduction frequency from 3 independent experiments, with error bars indicating the standard deviation.

ICT1 suppresses the growth defect in C. crescentus Δ*ssrA* cells

Previous characterization of *trans*-translation and ArfB activity in *C. crescentus* showed that cells lacking *trans*-translation grow slowly (12), and over-expression of ArfB partially suppresses this growth defect (21). To determine if ICT1 can perform the same function as ArfB in this assay, the growth rate of the Δ*ssrA* strain expressing ICT1 was measured and compared to control strains. Δ*ssrA* cells expressing ICT1 cells grew at a rate similar to Δ*ssrA* cells expressing ArfB, and significantly faster than Δ*ssrA* cells ($p < 0.01$) (Figure 3-4). These results indicate that ICT1 activity supports the same rate of growth as ArfB activity, and suggest that human ICT1 is functionally interchangeable with ArfB in *C. crescentus*.

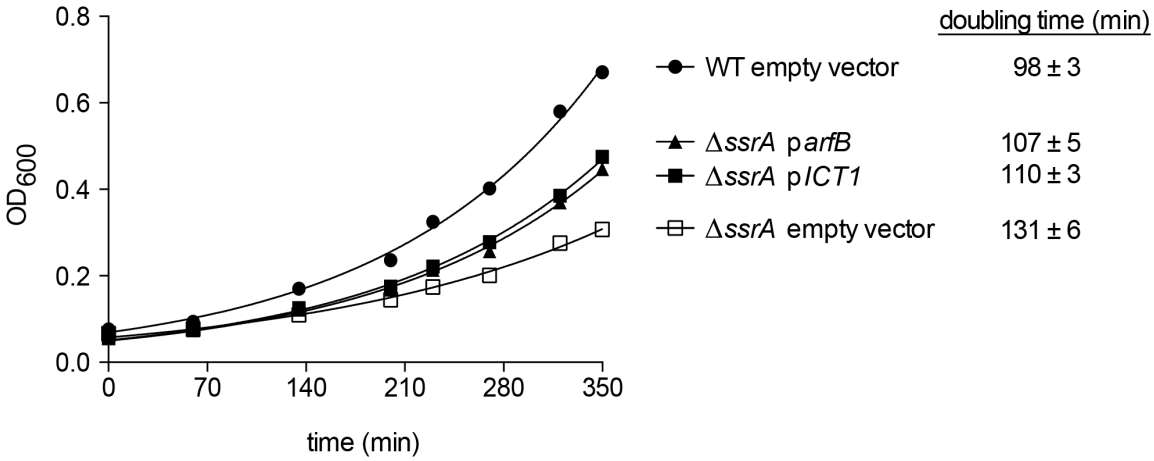

Figure **3-4**: Expression of ICT1 partially complements the growth defect of Δ*ssrA* cells. Growth of wild-type cells, Δ*ssrA* cells, or Δ*ssrA* cells expressing ArfB or ICT1 from a plasmid was monitored during exponential phase. The average doubling times (± standard deviation) from ≥3 experiments are shown.

ArfB rescues human cells when ICT1 is silenced

Mutant versions of ICT1 that lack the GGQ motif required for peptidyl-tRNA hydrolysis do not support viability in human cells when the endogenous ICT1 is silenced (26), indicating that ICT1-mediated peptidyl-tRNA hydrolysis is essential. If the essential activity of ICT1 is rescue of nonstop ribosomes, *C. crescentus* ArfB might be able to support viability in the absence of ICT1. To test this possibility, a gene encoding the N-terminal sequence of ICT1 containing the mitochondrial localization signal (33) fused to ArfB was constructed. HEK293 cells were transfected with a vector to express ArfB or ICT1, or with an empty vector. Eight hours later, these cells were transfected with siRNA that silenced ICT1 or with control siRNA, and after 6 days the number of living cells was determined. Consistent with previous results, ICT1 silencing decreased the number of viable cells, but cells expressing a version of ICT1 that was not silenced by the siRNA were not affected. Expression of ArfB prior to ICT1 silencing also prevented loss of viability. Cells expressing ArfB prior to ICT1 silencing grew to significantly higher numbers than cells transfected with vector alone ($p < 0.0001$) (Figure 4-5). There was no significant difference in viable cell number when cells were rescued with ArfB versus ICT1 ($p = 0.75$). These results indicate that ArfB can perform the essential function of ICT1 in human cells.

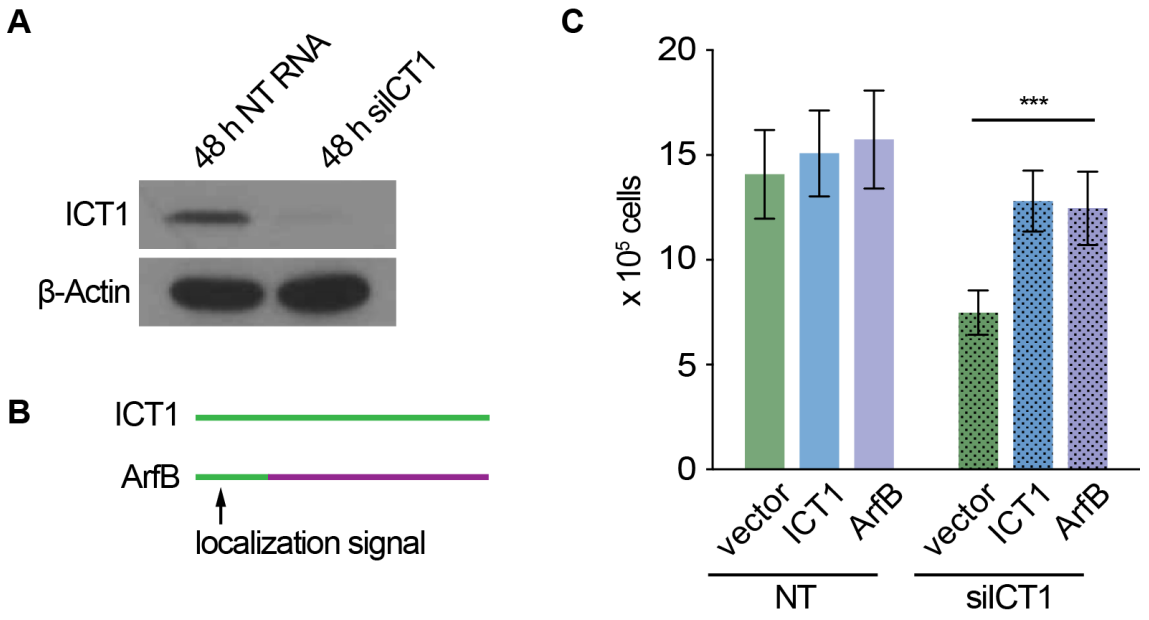

Figure **3-5**: *C. crescentus* ArfB rescues human cells from ICT1 silencing. Viability of HEK293 cells expressing ICT1 or ArfB was determined after silencing endogenous ICT1. (A) Western bot showing depletion of ICT1 after silencing with siICT1. Non-targeting siRNA (siNT) was used as a negative control. (B) Schematic diagram showing ArfB with ICT1 localization signal that was used for rescue. (C) Column graphs showing average viable cell numbers from 5 independent experiments. Error bars indicate standard deviation. *** indicates $p < 0.0001$.

Materials and Methods

Bacterial Growth

Strains are described in Table 3-1. *C. crescentus* strains were grown at 30 ˚C (34) in peptone-yeast extract (PYE) medium supplemented with tetracycline (2 µg/ml), streptomycin (50 µg/mL), spectinomycin (100 µg/mL), kanamycin (20 µg/mL), or xylose (0.3%) where appropriate. Growth was monitored by measuring optical density at 600 nm.

Table 3-1: Strains, plasmids, oligonucleotides, RNAs

Name	**Description**	**Reference**
Strains		
CB15N	Wild-type *C. crescentus*	(34)
CB15NΔ*ssrA*	In-frame deletion of *ssrA*	(12)
KCK426	*E. coli* BL21(DE3) carrying pET28*arfB*	(21)
KCK428	CB15N *arfB*::Ω with Kan marker 22.4 kb downstream	(21)
Plasmids		
pET28*arfB*	Used to express *C. crescentus* ArfB from T7 promoter	(21)
pET28*ICT1*	Used to express *H. sapiens* ICT1 from T7 promoter	This study
pMSCV*arfB*	pMSCVneo expressing ArfB with localization signal	This study
pMSCV*ICT1*	pMSCVneo expressing ICT1	This study
p*arfB*	encodes ArfB under the control of a xylose promoter	(21)
p*ICT1*	encodes ICT1 under the control of a xylose promoter	This study
Primers		

HAF_T7	CGAAATTAATACGACTCACTATAGGG	(21)
DHFR_NS_R	AAACCCCTCCGTTTAGAGAGGGGTTTTGCTAGTATCCGCCGCTCCAGAATCTCAAAGCAA	(21)
DHFR_stop_0	TTAACCCCTCCGTTTTAGAGAGGGGTTAATTGCTAGCCGCCGCTCCAGAATCTCAAAGCA	This study
DHFR_stop_6	AAACCCTTACTCCGTAGAGAGTAAGGGTTTTGCTAGCCGCCGCTCCAGAATCTCAAAGCA	This study
DHFR_stop_14	AAAAACCCCTCCGTTTAAGAGAGGGGTTTTGCTAGTATCCGCCGCTCCAGAATCTCAAAG	This study
DHFR_stop_33	AAAACCCCTCCGTTTAGAGAGGGGTTTTGCTAGTTACCGCCGCTCCAGAATCTCAAAGCA	This study
RNAs		
siNT	AGGUAGUGUAAUCGCCUUG dtdt	Eurofins Genomics
siICT1	GCCGCUAUCAGUUCCGGAA dtdt	(26)

Plasmid construction

To construct pET28*ICT1* for expression and purification of mature ICT1, the coding sequence (codon-optimized for *E. coli*) ICT1 lacking the mitochondrial localization signal was purchased as a gBlock Gene Fragment (IDT) and inserted into pET28b by Gibson assembly (35). Construction of p*arfB* (formerly p*CC1214*) has been described previously [26]. p*ICT1* was constructed by digesting pET28*ICT1* with NdeI and BamHI and ligating the resulting fragment into p*arfB* digested with the same enzymes. pMCSV*ICT1* was constructed by Gibson assembly of the human ICT1 sequence into the EcoRI and NotI sites of pMSCVneo. Alternative codons were selected for the region of ICT1 targeted by the siRNA to ensure that only endogenous ICT1

would be silenced. pMCSV*arfB* was similarly constructed by Gibson assembly of a gBlock® Gene Fragment into pMSCVneo at the EcoRI and NotI sites. The *arfB* construct encodes the 62 residue N-terminal extension of human ICT1 to target it to mitochondria followed by the 142 residues of *C. crescentus* ArfB sequence codon-optimized for expression in human cells. The ArfB coding sequence was codon-optimized for expression in human cells.

Purification of ArfB and ICT1

Purification of *C. crescentus* ArfB has been described previously (21). ICT1 was purified using a similar protocol. Strain KCK477 was grown to OD600 ~ 0.8 and expression was induced by addition of isopropyl-ß-D-thiogalactopyranoside (IPTG) to 1 mM. Cells were grown for 3 h at 37 ˚C, harvested by centrifugation at 4 ˚C, and resuspended in 30 ml lysis buffer (6 M guanidine hydrochloride, 20 mM sodium phosphate, 400 mM NaCl) [pH 7.8]. Cells were lysed by sonication and the lysates were cleared by centrifugation at 11,000 *g* for 30 min and applied to a column packed with 500 µl Ni-nitrilotriacetic acid (NTA) agarose (Qiagen) slurry equilibrated in DB buffer (8 M urea, 20 mM sodium phosphate 500 mM NaCl) [pH 7.8]. The column was washed 3X by rocking with 10 bed volumes DB buffer, washed 3X by rocking with 20 bed volumes DW buffer (8 M urea, 20 mM sodium phosphate, 500 mM NaCl) [pH 6.0], and washed 3X with 20 bed volumes of DW buffer [pH 5.3]. Protein was eluted in 1 ml fractions in elution buffer (8 M urea, 20 mM sodium phosphate, 500 mM NaCl) [pH 4.0]. Fractions containing ICT1 were dialyzed against ICT1 dialysis buffer (10 mM HEPES [pH 7.6], 150 mM NaCl). The 6 histidine tag was cleaved with Thrombin CleanCleave Kit (Sigma) at 4 ˚C for 3 h according to the manufacturer's instructions. Residual 6x-His tagged protein was removed by incubation with Ni-NTA agarose.

Peptidyl-tRNA hydrolysis assays

Peptidyl-tRNA hydrolysis by ArfB, ICT1, and release factors was assayed using the PURExpressΔRF1,2,3 kit (New England Biolabs). Template used for in vitro transcription and translation was generated by PCR using the primers listed in Table 1. Each template was designed to produce an mRNA with a stem-loop at the 3' end to limit exonuclease activity during the reaction. PURExpressΔRF1,2,3 kit components were mixed according to the manufacturer's instructions and incubated with 200 nM ArfB, 200 nM ICT1, or 100 nM RF1, RF2 and RF3 for 1 h at 37 ˚C. Anti-ssrA oligonucleotide was added to 5 µM to inhibit any *trans*-translation activity from tmRNA in the kit components. Samples were precipitated in 20 µl cold acetone, resuspended in loading buffer pH 6.5 (5 mM sodium bisulfite, 50 mM MOPS [morphonlinepropanesulfonic acid], 50 mM Tris, 1 µM EDTA, 0.1% SDS, 5% glycerol, 0.01% xylene cyanol, and 0.01% bromophenol blue), heated to 65 ˚C for 5 minutes, and resolved on Bis-Tris gels with MOPS running buffer (250 mM MOPS, 250 mM Tris, 5 mM EDTA and 0.5% SDS).

Phage transduction

ΦCR30 lysate was prepared from strain KCK428 as described previously (32). The resulting lysate was used to transduce wild-type or Δ*ssrA* cells harboring p*ICT1*, p*arfB*, or empty vector. Overnights of each strain were grown in PYE supplemented with tetracycline and xylose to mid log phase. 25 µl ΦCR30 prepared from KCK428 was added and cultures were incubated at 30 ˚C for 2.5 h with shaking. Cells were then plated on PYE with kanamycin and xylose to select for transductants. The resulting colonies were tested for spectinomycin and streptomycin resistance to determine the frequency with which *arfB:aadA* co-transduced.

ICT1 silencing

Pre-annealed non-targeting control siNT, or targeting siICT1(26) RNAs were purchased from Eurofins MWG Operon. To demonstrate efficient knockdown, 7.5×10^5 HEK293 cells were seeded in a 6-well plate and transfected once every 24 h with siNT and siICT1 as follows: a mixture containing 90 µl serum-free DMEM (Corning), 3.4 µl siRNA (20 µM stock), and 4 µl TransIT-siQUEST (Mirus Bio) was incubated at room temperature for 30 min and added to the cells. After 48 h, cells were lysed by addition of buffer containing 150 mM sodium chloride, 50 mM Tris [pH 8.0], 5 mM EDTA, 2 mM phenylmethylsulfonyl fluoride, 2 mM sodium orthovanadate, 10 mM sodium fluoride, and 1% Igepal TM CA-360 (USB). The efficacy of ICT1 silencing was then determined by western blot using polyclonal mouse anti-human ICT1 (Sigma).

In vitro translation and peptidyl-tRNA hydrolysis assays

CC1214 was assayed for peptidyl hydrolysis activity using the PURExpress system (New England Biolabs) similar to previous assays (36)with some modifications. Nonstop DHFR was amplified using primers HAF_T7 and UTR_DHFR_FL and used as template for the reaction. After 1 h incubation at 37 °C, CC1214 or CC1214G30A was added to a final concentration of 200 nM. A control sample was treated with 70 µg/ml puromycin. After 1 h incubation at 37 °C, total protein was precipitated by addition of acetone, resuspended in sample loading buffer (5 mM sodium bisulfite, 50 mM MOPS, 50 mM tris base, 1 µM EDTA, 0.1% SDS, 5% glycerol, 0.01% xylene cyanol, 0.01% bromophenol blue), and resolved on a bis-tris gel using MOPS running buffer.

ICT1 rescue in HEK293 cells

HEK293 cells (ATCC) were seeded in 6-well plates at 5×10^4 cells per well and allowed to adhere for 18 h. Cells were transfected by combining 1 µg pMSCV vector, pMSCV*ICT1*, or pMSCV*arfB*, 90 µl serum-free DMEM, and 3.8 µl TransIT-293 (Mirus Bio) and allowing the mixture to incubate for 30 min at room temperature. 8 h after transfecting with plasmid, cells were transfected with siNT or siICT1 according to the siRNA transfection protocol described in the previous section. Viable cell numbers were determined by 0.4% trypan blue staining of trypsinized cells 6 days after silencing.

Discussion

Rescue of ribosomes from nonstop translation complexes is a critical function for most bacteria, and the results presented here indicate that rescue of mitochondrial ribosomes is also essential in some eukaryotes. *C. crescentus* cells can survive without tmRNA and SmpB because they have ArfB to rescue ribosomes in the absence of *trans*-translation (21). We have previously shown that *C. crescentus* ArfB can hydrolyze peptidyl-tRNA from nonstop ribosomes in vitro (21). The data described here show that ArfB is also active on ribosomes stalled with a full codon in the A site or with 6 nucleotides past the A site, but longer mRNA extensions strongly inhibit ArfB activity. This substrate specificity is similar to that observed for tmRNA-SmpB (37-39), and indicates that ArfB is unlikely to interfere with translation elongation or with ribosomes paused during translation of full-length mRNAs. Instead, ArfB activity is consistent with a role in rescuing ribosomes that have translated to the 3' end of an mRNA without terminating, ribosomes that have stalled after cleavage of the mRNA in the A site by a nuclease such as RelE (40), and stalled ribosomes that have had the 3' portion of the mRNA removed by exonuclease activity (39,41).

Several lines of evidence demonstrate that human ICT1 is a ribosome rescue factor like ArfB, and not a non-specific release factor that can act on ribosomes stalled in the middle of an mRNA. First, the specificity of ICT1 in vitro for nonstop ribosomes or ribosomes with short mRNA extensions past the A site is similar to that of ArfB. Second, ICT1 can functionally replace ArfB in *C. crescentus.* Expression of ICT1 suppresses the synthetic lethality of deleting *ssrA* and *arfB*, and over-expression of ICT1 increases the growth rate in Δ*ssrA* cells to the same extent as over-expression of ArfB. Third, expression of ArfB in human cells suppresses the lethal effects of silencing ICT1, indicating that ArfB can functionally replace ICT1 in human cells. Finally, ICT1 would have little opportunity to act as a non-specific release factor during translation of intact

transcripts in the mitochondria because mammalian mitochondrial transcripts are polyadenylated with ~50 nucleotides (42-47). Based on the substrate specificity of ICT1 in vitro, this poly(A) tail would block ICT1 activity unless the mRNA was truncated, so ICT1 substrates for in vivo are likely to be nonstop complexes. Because ICT1 is essential in human cells (26,27), these results suggest that ribosome rescue in mitochondria is essential for human cell viability. The activity of ICT1 as a rescue factor and not a non-specific release factor would also explain why ICT1 does not interfere with translation elongation in mitochondria.

ICT1 substrate specificity has important implications for translation termination in mitochondria. Two human mitochondrial genes end in an AGG (*ND6*) or AGA (*COI*) codon. There are no cognate mitochondrial tRNAs for these codons and the mitochondrial release factor mtRF1a is unable terminate translation at AGA or AGG (48), so it is unclear how translation is terminated for these genes. ICT1 has been proposed to function as the termination factor at these codons based on analysis of activity in vitro on ribosomes stalled with an mRNA extending up to 14 nucleotides past the A site (30,31). Our data show that ICT1 activity is greatly reduced on ribosomes stalled with an mRNA extending 14 nucleotides past the A site, and ICT1 activity is completely absent when the mRNA extends 33 nucleotides past the A site. Because the *COI* AGA codon is 72 nucleotides from the end of the transcript and the *ND6* AGG codon is 500-550 nucleotides from the end of the transcript (42,45), ICT1 should have no activity on these transcripts in either their unmodified or polyadenylated form. In addition, ICT1 can support viability in *C. crescentus*, so it cannot have an intrinsic ability to terminate translation at AGA or AGG because these codons encode arginine in bacteria. Likewise, ArfB does not recognize AGA or AGG in *C. crescentus*, so the ability of ArfB to replace ICT1 in human mitochondria suggests that termination at AGA or AGG in the middle of a transcript is not an essential function for ICT1. One possible mechanism for both ICT1 and ArfB to terminate translation at these codons is that stalling of the ribosomes leads to truncation of the mRNA 3’ of the ribosome, thereby

producing a substrate for the rescue factors. A second possible mechanism for termination at AGA or AGG by ICT1 and ArfB would be that mitochondrial ribosomes might respond differently when they stall in the middle of an mRNA than do bacterial ribosomes. Mitochondrial ribosomes are descended from bacterial ribosomes, but are highly specialized for translating a small number of mRNAs encoded in the mitochondrial genome. The decoding center and peptidyl transfer center of mitochondrial ribosomes are very similar to bacterial ribosomes, but other architectural features are highly diverged (49). For example, mammalian mitochondria have dramatically reduced rRNAs and lack 5S rRNA, but contain 36 proteins not found in bacteria and incorporate a tRNA as a structural component of the large subunit (50). It is possible that this different architecture causes mitochondrial ribosomes to adopt a conformation that promotes rescue by ICT1 and ArfB when they are stalled in the middle of an mRNA. If ICT1 does not terminate translation at these codons, one of the other members of the mitochondrial RF family might perform this function.

Mitochondria descend from a progenitor of α-proteobacteria (51,52), the bacterial class that includes *C. crescentus*. Most α-proteobacterial species contain both *trans*-translation and ArfB, and some protist mitochondria encode *ssrA* and *smpB* (4), suggesting that the primordial mitochondrion had both ribosome rescue systems. Why did mitochondria keep ArfB and discard *trans*-translation, whereas almost all bacteria have kept *trans*-translation whether they have an alternative rescue factor or not? Mitochondria encode only 13 proteins, all of which are integral membrane proteins. Perhaps this limited proteome decreases the selective advantages of *trans*-translation, for example by enabling proteases to recognize incomplete versions of proteins without the tmRNA-encoded tag. Alternatively, the constraints of importing factors encoded in the nucleus might have favored ICT1 over tmRNA-SmpB, because ICT1 acts as a single protein. There is at least one eukaryote that is likely to use trans-translation to rescue nonstop ribosomes in mitochondria. Oomyceta, a lineage of fungal eukaryotes, import SmpB into their mitochondria,

and encode tmRNA on the mitochondrial genome (4). Mitochondrial genomes have undergone severe gene loss. Retention of tmRNA in Oomyceta mitochondrial genomes where it can be transcribed within the organelle also suggests a barrier to tmRNA import. Interestingly, some plants encode an ArfB/ICT1 homolog with a chloroplast-targeting signal, (Q84JF2, e.g.) suggesting that ribosome rescue activity may be found in other organelles, and that the ArfB-type ribosome rescue may be generally favored in eukaryotic organelles.

References

1. Schmeing TM, Ramakrishnan V. What recent ribosome structures have revealed about the mechanism of translation. Nature. Nature Publishing Group; 2009 Oct 29;461(7268):1234–42.

2. Ito K, Uno M, Nakamura Y. A tripeptide “anticodon” deciphers stop codons in messenger RNA. Nature. Nature Publishing Group; 2000 Feb 10;403(6770):680–4.

3. Keiler KC. Mechanisms of ribosome rescue in bacteria. Nat Rev Microbiol. Nature Publishing Group; 2015 May;13(5):285–97.

4. Hudson CM, Lau BY, Williams KP. Ends of the line for tmRNA-SmpB. Front Microbiol. Frontiers; 2014;5(93):421.

5. Keiler KC, Feaga HA. Resolving nonstop translation complexes is a matter of life or death. Journal of Bacteriology. American Society for Microbiology; 2014 Jun;196(12):2123–30.

6. Huang C, Wolfgang MC, Withey J, Koomey M, Friedman DI. Charged tmRNA but not tmRNA-mediated proteolysis is essential for Neisseria gonorrhoeae viability. EMBO J. EMBO Press; 2000 Mar 1;19(5):1098–107.

7. Ramadoss NS, Zhou X, Keiler KC. tmRNA is essential in Shigella flexneri. Herman C, editor. PLoS ONE. Public Library of Science; 2013;8(2):e57537.

8. Thibonnier M, Thiberge J-M, De Reuse H. Trans-translation in Helicobacter pylori: essentiality of ribosome rescue and requirement of protein tagging for stress resistance and competence. Ahmed N, editor. PLoS ONE. Public Library of Science; 2008;3(11):e3810.

9. Svetlanov A, Puri N, Mena P, Koller A, Karzai AW. Francisella tularensis tmRNA system mutants are vulnerable to stress, avirulent in mice, and provide effective immune protection. Molecular Microbiology. Blackwell Publishing Ltd; 2012 Jul;85(1):122–41.

10. Julio SM, Heithoff DM, Mahan MJ. ssrA (tmRNA) plays a role in Salmonella enterica serovar Typhimurium pathogenesis. Journal of Bacteriology. American Society for Microbiology (ASM); 2000 Mar;182(6):1558–63.

11. Shin J-H, Price CW. The SsrA-SmpB ribosome rescue system is important for growth of Bacillus subtilis at low and high temperatures. Journal of Bacteriology. American Society for Microbiology; 2007 May;189(10):3729–37.

12. Keiler KC, Shapiro L. TmRNA is required for correct timing of DNA replication in Caulobacter crescentus. Journal of Bacteriology. American Society for Microbiology (ASM); 2003 Jan;185(2):573–80.

13. Oh BK, Apirion D. 10Sa RNA, a small stable RNA of Escherichia coli, is functional. Mol Gen Genet. 1991 Sep;229(1):52–6.

14. Chadani Y, Ono K, Ozawa S-I, Takahashi Y, Takai K, Nanamiya H, et al. Ribosome rescue by Escherichia coli ArfA (YhdL) in the absence of trans-translation system. Molecular Microbiology. Blackwell Publishing Ltd; 2010 Nov;78(4):796–808.

15. Handa Y, Inaho N, Nameki N. YaeJ is a novel ribosome-associated protein in Escherichia coli that can hydrolyze peptidyl-tRNA on stalled ribosomes. Nucleic Acids Research. 2011 Mar 19;39(5):1739–48.

16. Chadani Y, Ono K, Kutsukake K, Abo T. Escherichia coli YaeJ protein mediates a novel ribosome-rescue pathway distinct from SsrA- and ArfA-mediated pathways. Molecular Microbiology. Blackwell Publishing Ltd; 2011 May;80(3):772–85.

17. Chadani Y, Ito K, Kutsukake K, Abo T. ArfA recruits release factor 2 to rescue stalled ribosomes by peptidyl-tRNA hydrolysis in Escherichia coli. Molecular Microbiology. Blackwell Publishing Ltd; 2012 Oct;86(1):37–50.

18. Shimizu Y. ArfA recruits RF2 into stalled ribosomes. J Mol Biol. 2012 Nov 2;423(4):624–31.

19. Kurita D, Chadani Y, Muto A, Abo T, Himeno H. ArfA recognizes the lack of mRNA in the mRNA channel after RF2 binding for ribosome rescue. Nucleic Acids Research. Oxford University Press; 2014 Dec 1;42(21):13339–52.

20. Zeng F, Jin H. Peptide release promoted by methylated RF2 and ArfA in nonstop translation is achieved by an induced-fit mechanism. RNA. Cold Spring Harbor Lab; 2016 Jan;22(1):49–60.

21. Feaga HA, Viollier PH, Keiler KC. Release of nonstop ribosomes is essential. MBio. American Society for Microbiology; 2014;5(6):e01916.

22. Kogure H, Handa Y, Nagata M, Kanai N, Guntert P, Kubota K, et al. Identification of residues required for stalled-ribosome rescue in the codon-independent release factor YaeJ. Nucleic Acids Research. 2014 Mar 11;42(5):3152–63.

23. Gagnon MG, Seetharaman SV, Bulkley D, Steitz TA. Structural basis for the rescue of stalled ribosomes: structure of YaeJ bound to the ribosome. Science. American Association for the Advancement of Science; 2012 Mar 16;335(6074):1370–2.

24. Passos DO, Doma MK, Shoemaker CJ, Muhlrad D, Green R, Weissman J, et al. Analysis of Dom34 and its function in no-go decay. Mol Biol Cell. American Society for Cell Biology; 2009 Jul;20(13):3025–32.

25. Chen L, Muhlrad D, Hauryliuk V, Cheng Z, Lim MK, Shyp V, et al. Structure of the Dom34-Hbs1 complex and implications for no-go decay. Nature Structural & Molecular Biology. Nature Publishing Group; 2010 Oct;17(10):1233–40.

26. Richter R, Rorbach J, Pajak A, Smith PM, Wessels HJ, Huynen MA, et al. A functional peptidyl-tRNA hydrolase, ICT1, has been recruited into the human mitochondrial ribosome. EMBO J. 2010 Feb 25;29(6):1116–25.

27. Kogure H, Hikawa Y, Hagihara M, Tochio N, Koshiba S, Inoue Y, et al. Solution structure and siRNA-mediated knockdown analysis of the mitochondrial disease-related protein C12orf65. Proteins. Wiley Subscription Services, Inc., A Wiley Company; 2012 Nov;80(11):2629–42.

28. Chrzanowska-Lightowlers ZM, Lightowlers RN. Response to “Ribosome Rescue and Translation Termination at Non-standard Stop Codons by ICT1 in Mammalian Mitochondria.” Larsson N-G, editor. PLoS Genet. 2015 Jun 18;11(6):e1005227–4.

29. Takeuchi N, Nierhaus KH. Response to the Formal Letter of Z. Chrzanowska-Lightowlers and R. N. Lightowlers Regarding Our Article "Ribosome Rescue and Translation Termination at Non-Standard Stop Codons by ICT1 in Mammalian Mitochondria". Larsson N-G, editor. PLoS Genet. Public Library of Science; 2015 Jun;11(6):e1005218.

30. Lind C, Sund J, Aqvist J. Codon-reading specificities of mitochondrial release factors and translation termination at non-standard stop codons. Nat Commun. Nature Publishing Group; 2013;4:2940.

31. Akabane S, Ueda T, Nierhaus KH, Takeuchi N. Ribosome Rescue and Translation Termination at Non-Standard Stop Codons by ICT1 in Mammalian Mitochondria. Larsson N-G, editor. PLoS Genet. 2014 Sep 18;10(9):e1004616–14.

32. West L, Yang D, Stephens C. Use of the Caulobacter crescentus genome sequence to develop a method for systematic genetic mapping. Journal of Bacteriology. American Society for Microbiology (ASM); 2002 Apr;184(8):2155–66.

33. Handa Y, Hikawa Y, Tochio N, Kogure H, Inoue M, Koshiba S, et al. Solution structure of the catalytic domain of the mitochondrial protein ICT1 that is essential for cell vitality. J Mol Biol. 2010 Nov 26;404(2):260–73.

34. Evinger M, Agabian N. Envelope-associated nucleoid from Caulobacter crescentus stalked and swarmer cells. Journal of Bacteriology. American Society for Microbiology (ASM); 1977 Oct;132(1):294–301.

35. Gibson DG, Young L, Chuang R-Y, Venter JC, Hutchison CA, Smith HO. Enzymatic assembly of DNA molecules up to several hundred kilobases. Nat Methods. Nature Publishing Group; 2009 May;6(5):343–5.

36. Ramadoss NS, Alumasa JN, Cheng L, Wang Y, Li S, Chambers BS, et al. Small molecule inhibitors of trans-translation have broad-spectrum antibiotic activity. Proc Natl Acad Sci USA. National Acad Sciences; 2013 Jun 18;110(25):10282–7.

37. Ivanova N, Pavlov MY, Felden B, Ehrenberg M. Ribosome rescue by tmRNA requires truncated mRNAs. J Mol Biol. 2004 Apr 16;338(1):33–41.

38. Moore SD, Sauer RT. Ribosome rescue: tmRNA tagging activity and capacity in Escherichia coli. Molecular Microbiology. Blackwell Science Ltd; 2005 Oct;58(2):456–66.

39. Hayes CS, Sauer RT. Cleavage of the A site mRNA codon during ribosome pausing

provides a mechanism for translational quality control. Mol Cell. 2003 Oct;12(4):903–11.

40. Pedersen K, Zavialov AV, Pavlov MY, Elf J, Gerdes K, Ehrenberg M. The bacterial toxin RelE displays codon-specific cleavage of mRNAs in the ribosomal A site. Cell. 2003 Jan 10;112(1):131–40.

41. Garza-Sánchez F, Shoji S, Fredrick K, Hayes CS. RNase II is important for A-site mRNA cleavage during ribosome pausing. Molecular Microbiology. Blackwell Publishing Ltd; 2009 Sep;73(5):882–97.

42. Bobrowicz AJ, Lightowlers RN, Chrzanowska-Lightowlers Z. Polyadenylation and degradation of mRNA in mammalian mitochondria: a missing link?: Table 1. Biochm Soc Trans. 2008 Jun 1;36(3):517–9.

43. Chang JH, Tong L. Mitochondrial poly(A) polymerase and polyadenylation. Biochim Biophys Acta. 2012 Sep;1819(9-10):992–7.

44. Tomecki R, Dmochowska A, Gewartowski K, Dziembowski A, Stepien PP. Identification of a novel human nuclear-encoded mitochondrial poly(A) polymerase. Nucleic Acids Research. Oxford University Press; 2004;32(20):6001–14.

45. Slomovic S, Laufer D, Geiger D, Schuster G. Polyadenylation and degradation of human mitochondrial RNA: the prokaryotic past leaves its mark. Mol Cell Biol. American Society for Microbiology; 2005 Aug;25(15):6427–35.

46. Nagaike T, Suzuki T, Katoh T, Ueda T. Human mitochondrial mRNAs are stabilized with polyadenylation regulated by mitochondria-specific poly(A) polymerase and polynucleotide phosphorylase. J Biol Chem. American Society for Biochemistry and Molecular Biology; 2005 May 20;280(20):19721–7.

47. Ojala D, Attardi G. Expression of the mitochondrial genome in HeLa cells. XIX. Occurrence in mitochondria of polyadenylic acid sequences, “free” and covalently linked to mitochondrial DNA-coded RNA. J Mol Biol. 1974 Jan 15;82(2):151–74.

48. Nozaki Y, Matsunaga N, Ishizawa T, Ueda T, Takeuchi N. HMRF1L is a human mitochondrial translation release factor involved in the decoding of the termination codons UAA and UAG. Genes Cells. Blackwell Publishing Inc; 2008 May;13(5):429–38.

49. Ott M, Amunts A, Brown A. Organization and Regulation of Mitochondrial Protein Synthesis. Annu Rev Biochem. Annual Reviews 4139 El Camino Way, PO Box 10139, Palo Alto, California 94303-0139, USA; 2016 Jan 18;85(1):annurev–biochem–060815–014334.

50. Amunts A, Brown A, Toots J, Scheres SHW, Ramakrishnan V. Ribosome. The structure of the human mitochondrial ribosome. Science. American Association for the Advancement of Science; 2015 Apr 3;348(6230):95–8.

51. Fitzpatrick DA, Creevey CJ, McInerney JO. Genome phylogenies indicate a meaningful alpha-proteobacterial phylogeny and support a grouping of the mitochondria with the Rickettsiales. Mol Biol Evol. Oxford University Press; 2006 Jan;23(1):74–85.

52. Andersson SG, Zomorodipour A, Andersson JO, Sicheritz-Pontén T, Alsmark UC, Podowski RM, et al. The genome sequence of Rickettsia prowazekii and the origin of mitochondria. Nature. Nature Publishing Group; 1998 Nov 12;396(6707):133–40.

Chapter 4

Investigating the role of *trans*-translation in quality control of cotranslational secretion

Abstract

Previous chapters have shown that rescue of nonstop ribosomes is essential. Drugs that target ribosome rescue have broad spectrum antimicrobial activity. What causes cell death after treatment with ribosome rescue inhibitors? Does inhibition of ribosome rescue affect other essential cellular process in addition to protein synthesis? Recently, it was shown that treatment with chloramphenicol, a translation inhibitor, resulted in rapid degradation of the SecY secretion channel. A model was proposed in which inhibition of ribosomes that are engaged in co-translational secretion resulted in jamming of SecY followed by rapid proteolysis of SecY. SecY degradation could contribute to cell death since membrane integrity depends on translocation of inner and outer membrane proteins through SecY. Treatment with the ribosome rescue inhibitor KKL-35 resulted in reduced cellular concentrations of the SecY protein. Entry into stationary phase was also accompanied by a decrease in SecY concentrations. Treatment with puromycin, a drug that nonspecifically terminates translation, also resulted in rapid reduction in SecY concentrations. More work is necessary to understand how SecY is regulated in response to drugs that target the ribosome.

Introduction

Approximately 25% of the bacterial proteome is secreted (1). The majority of these proteins are exported through the SecYEG translocase, which is universally conserved in bacteria (1). SecY forms the channel through which the unfolded polypeptide passes, and is essential for viability. SecE is also essential and provides structural support to the SecY channel (2). The role of SecG is currently unclear, and SecG is dispensable, except at low temperatures (3). Outer membrane and periplasmic proteins are targeted to SecYEG post-translationally, whereas inner membrane proteins are targeted cotranslationally (1). When proteins are inserted cotranslationally the ribosome is targeted to the translocase by the signal recognition particle (SRP), a ribonucleoprotein complex comprising 4.5S RNA and the GTPase Ffh (1,4). As soon as the signal sequence emerges from the ribosome, SRP delivers the ribosome to the signal receptor (FtsY), which binds to SecY (4). The large subunit of the ribosome then directly binds the SecY translocase (5,6). The energy of protein synthesis drives the peptide through the secretion channel. If a ribosome engaged with SecYEG becomes stalled, the secretion channel is thought to become unproductively jammed because it is the force of protein synthesis that pushes the peptide through the secretion channel, and the ribosome itself is too large to pass through the channel (7).

LacZ hybrid proteins have been used to study SecY-mediated secretion. Appending the LamB post-translational secretion signal to LacZ jams the secretion channel because LacZ folds in the cytoplasm and cannot be exported (7,8). When LamB-LacZ is over-expressed, the cellular concentration of SecY decreases, suggesting that jamming the secretion channel results in SecY degradation (7). When SecY is jammed, it is thought to be proteolyzed by the inner-membrane protease FtsH (9), because over-expression of YccA, an FtsH substrate and inhibitor of FtsH, stabilizes SecY when LamB-LacZ is over-expressed. Treatment of *Escherichia coli* with inhibitors of translation elongation, including chloramphenicol and tetracycline, also results in

degradation of SecY by FtsH (7). It has been proposed that SecY degradation is the direct result of the inhibited ribosome jamming the secretion channel, and that SecY degradation contributes to the action of translation inhibitors (7,10).

Ribosome rescue may have a special role at the bacterial membrane. Although actively translating ribosomes are distributed throughout the bacterial cell, tmRNA-SmpB localize in a helical pattern at the membrane in *Caulobacter crescentus* (11). This localization pattern has been reported for the SecY secretion channel in *Bacillus subtilis* (12). Positioning tmRNA-SmpB in a pattern that is consistent with SecY localization could ensure that *trans*-translation is available in a region where there are likely to be abundant nonstop ribosomes. Inner-membrane proteins that are cotranslationally secreted may be a major source of substrates for ribosome rescue. Inner-membrane proteins are often large, with extensive hydrophobic regions that may misfold during translation (13). Therefore, ribosome stalling may be more likely at the membrane, which may result in mRNA cleavage on the ribosome and recruitment of tmRNA-SmpB, ArfA, or ArfB. If ribosome rescue occurs frequently at the membrane, treatment with ribosome rescue inhibitors should result in jammed SecY channels that would be degraded by FtsH (Figure 4-1). Additionally, if a reduction in SecY contributes to the toxicity of translation inhibitors and ribosome rescue inhibitors then deletion of FtsH could result in decreased sensitivity to these drugs.

To test whether the cellular concentration of SecY protein in the cell decreases after inhibition of ribosome rescue, SecY was monitored after treatment with the ribosome rescue inhibitor KKL-35 (14,15). Treatment with KKL-35 or chloramphenicol resulted in reduction of SecY, suggesting that channel jamming may occur when ribosome rescue is inhibited. However, entry into stationary phase also resulted in a reduction in the cellular concentration of SecY. Treatment with high concentrations of puromycin, a structural analog of charged tRNA that terminates translation and should not result in jammed secretion channels, also resulted in

reduced levels of SecY per cell. These results suggest that inhibiting cellular growth results in SecY degradation, and that further studies are necessary to determine whether jamming of SecY occurs in response to translation elongation inhibitors as well as ribosome rescue inhibitors.

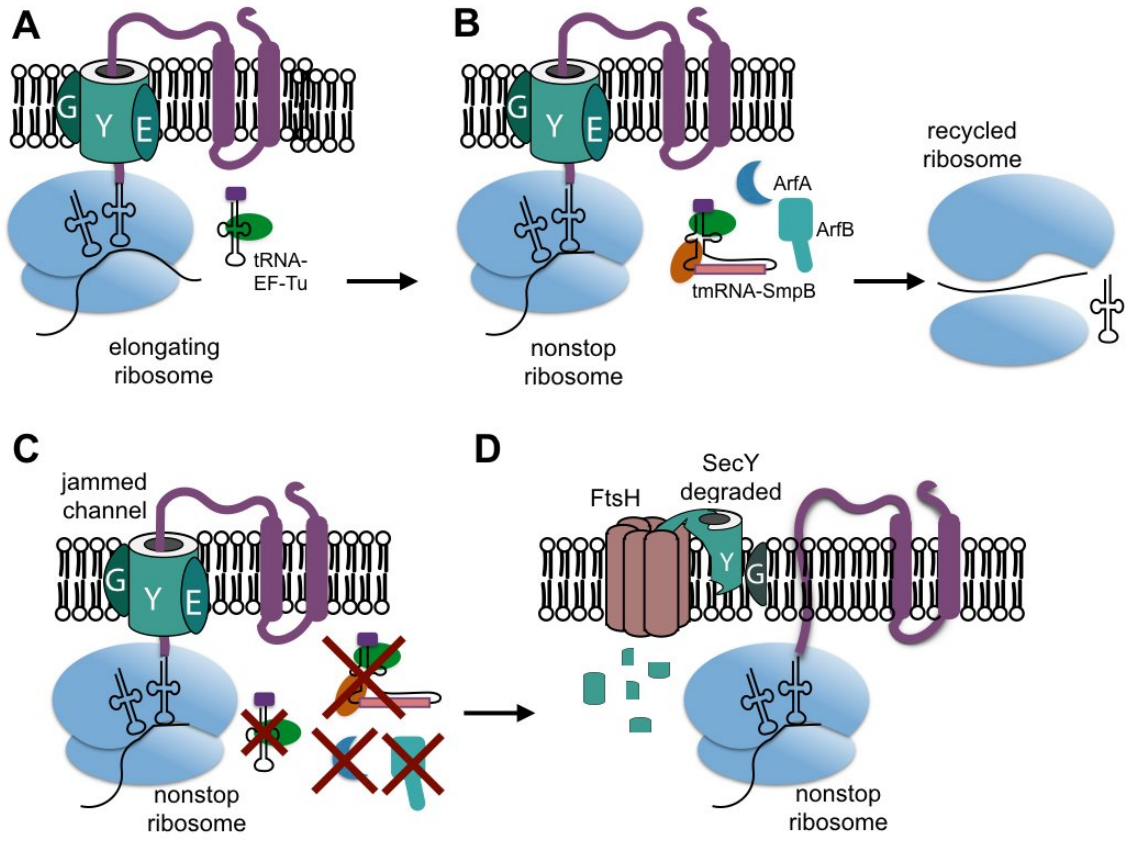

Figure **4-1**: Schematic of the role of ribosome rescue during cotranslational secretion and the effects of inhibition. (A) Inner membrane proteins are cotranslationally secreted. (B) Translation of a nonstop mRNA results in rescue by *trans*-translation (tmRNA-SmpB), ArfA, or ArfB. (C) Inhibition of elongation and rescue results in a jammed SecY channel. (D) The unproductive secretion channel is proteolyzed by FtsH. Adapted from Hayes & Keiler (2010) (13).

Results

Treatment with a translation inhibitor results in decreased concentration of SecY in cells

Chloramphenicol inhibits translation elongation by blocking peptidyl-transfer. To confirm previous results showing that treatment with chloramphenicol resulted in degradation of SecY (7), a strain was constructed to express M2-tagged SecY at the chromosomal locus. At 15 minute intervals following treatment with 10 µg/mL chloramphenicol or with vehicle, a sample was taken from each culture, and cell numbers were normalized by optical density (OD). Throughout the experiment, the volume of cells collected was normalized to the culture with the lowest OD at time zero. Cellular concentration of SecY was then monitored by western blot using anti-M2 antibodies. In agreement with previously reported results, treatment with 10 µg/mL chloramphenicol resulted in rapid degradation of M2-tagged SecY protein (figure 4-2) (7). Within 15 minutes after treatment, levels of SecY were reduced to 35% of the level present at the start of the experiment. After 30 minutes, levels of SecY had diminished to less than 10%.

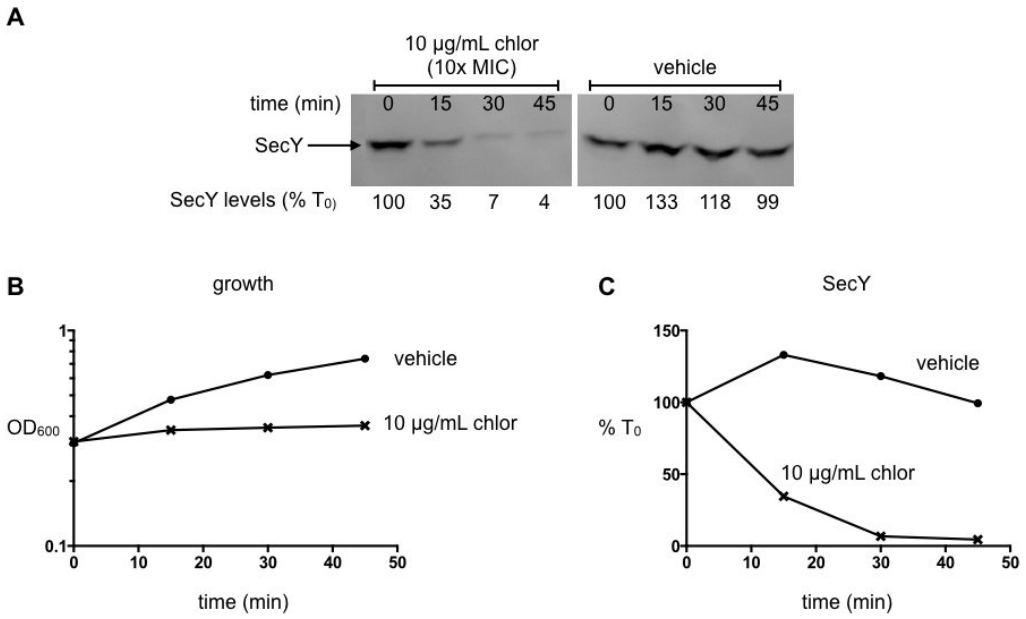

Figure **4-2**: The cellular concentration of SecY is reduced after treatment with chloramphenicol. (A) Western blot showing cellular concentration of SecY after treatment with 10 µg/mL chloramphenicol (dissolved in 50% ethanol) or with the same volume of 50% ethanol (vehicle). (B) Cellular growth monitored by optical density. (C) SecY concentration represented as a percent of SecY concentration at the start of the experiment.

Treatment with a ribosome rescue inhibitor results in decreased cellular SecY concentration

A decrease in the cellular concentration of SecY after treatment with chloramphenicol suggests that drugs that inhibit translation elongation may result in SecY channel jamming and degradation. These data also suggest that if ribosome rescue occurs frequently at the membrane, then inhibition of ribosome rescue may also result in reduction of SecY concentration in the cells. To test whether inhibiting ribosome rescue also results in a decreased SecY concentration, cellular concentrations of SecY were monitored after treatment with ribosome rescue inhibitor KKL-35. A dose dependent reduction in cellular SecY concentration was observed. Treatment

with 0.3 µM KKL-35 resulted in cellular concentrations of SecY that were similar to samples that were treated with vehicle only (Figure 4-3). At this concentration of KKL-35 cell numbers, as measured by OD, did continue to increase, indicating that growth had not been completely inhibited. In contrast, addition of KKL-35 to 1.5 or 3.0 µM resulted in growth inhibition after treatment and a rapid reduction in SecY concentration within 30 minutes after treatment. The cellular concentration of SecY decreased by more than half within 30 minutes of treatment with 1.5 µM KKL-35 and within 15 minutes of treatment with 3 µM KKL-35 (Figure 4-3).

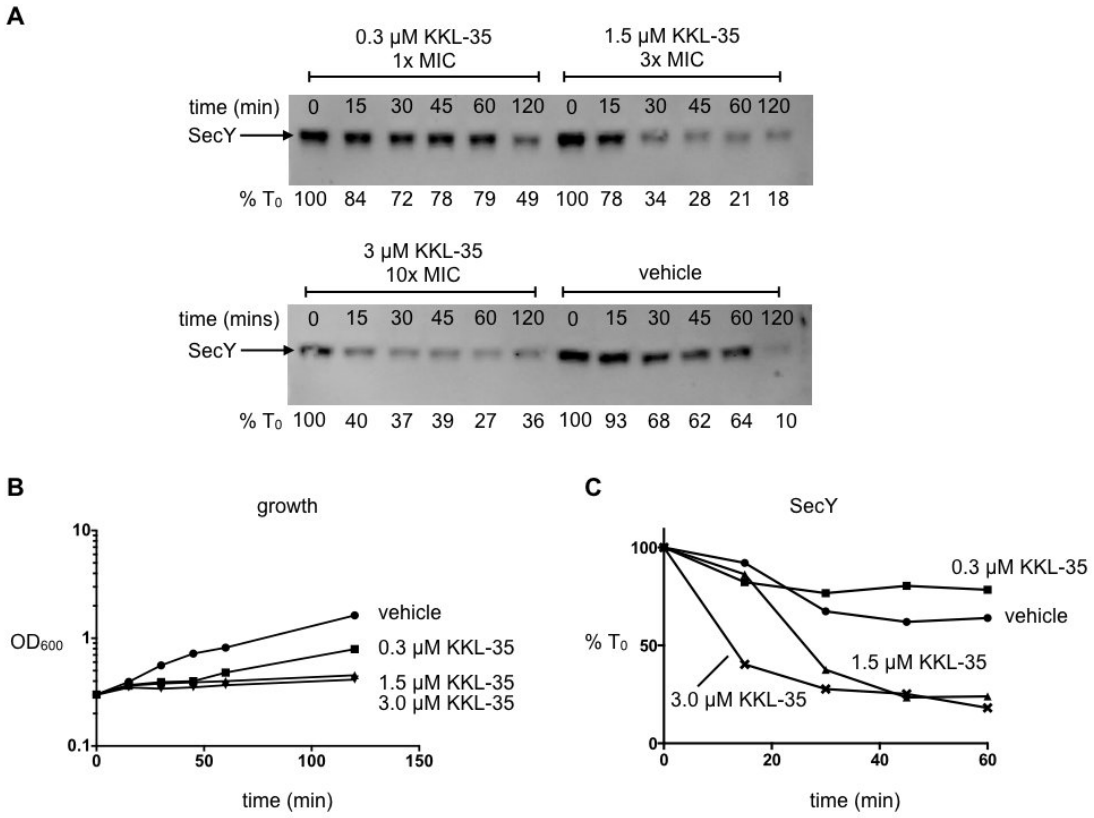

Figure **4-3**: The cellular concentration of SecY is reduced after treatment with KKL-35. (A) Western blots show cellular SecY concentrations after treatment with KKL-35 (dissolved in DMSO). A control culture was treated with DMSO only (vehicle). (B) Cellular growth measured by optical density is plotted as a function of time. (C) SecY concentration as a percent of concentration at the start of the experiment.

SecY concentration decreases as cells approach stationary phase

The cellular concentration of SecY in vehicle-treated cultures substantially decreased to less than 10% of the starting amount within 2 hours of monitoring (Figure 4-3). To further investigate the decrease in SecY as cells approach stationary phase, samples were collected over the course of 145 minutes in LB media or buffered EZ-rich media. As the cells approached stationary phase, the cellular concentration of SecY also began to decrease. After 145 minutes of growth, the concentration of SecY in both types of media had decreased to 15% or less of the concentration at the start of the experiment.

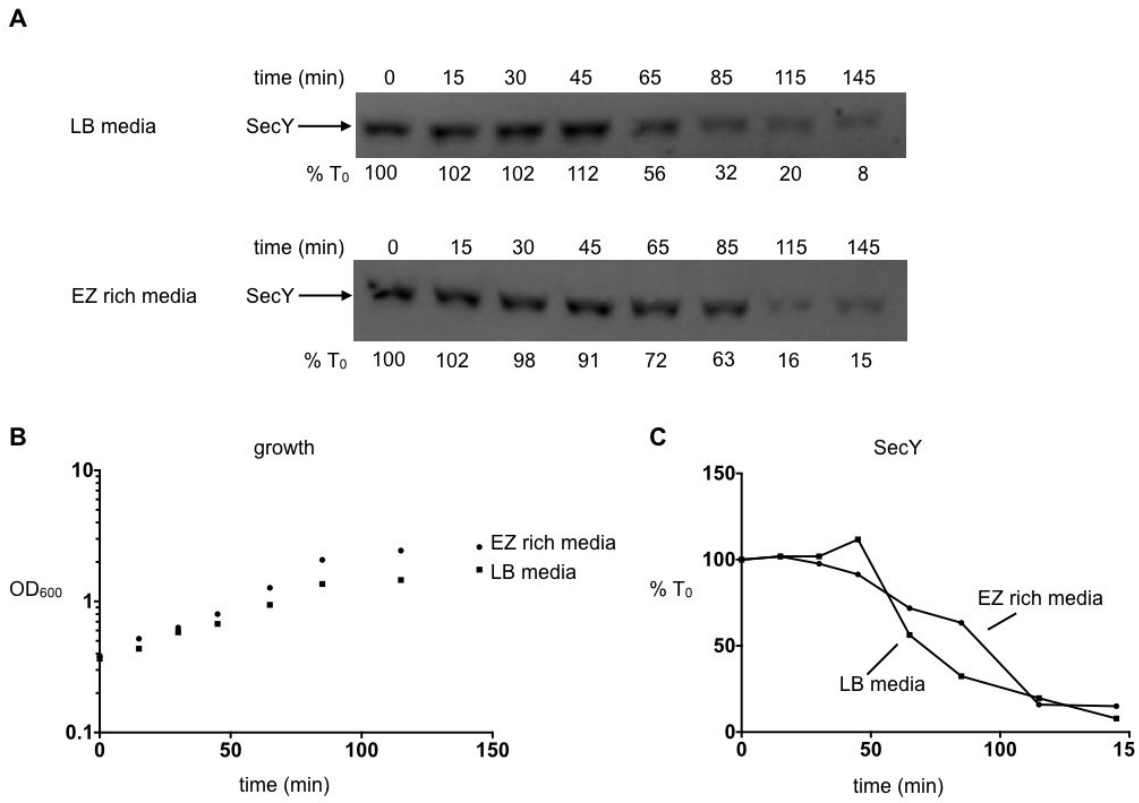

Figure **4-4**: SecY decreases as cells approach stationary phase. (A) Cellular SecY concentration monitored by western blot. (B) Growth of cells monitored by optical density. (C) Plot of SecY

concentration as a percent of SecY concentration at the start of the experiment.

Treatment with rifamycin results in a dose dependent effect on growth and SecY concentration

Reduction in cellular SecY concentration as the cells approach stationary phase suggests that the effect of chloramphenicol and KKL-35 may be the result of growth inhibition. However, it has been proposed that inhibiting transcription, which should also inhibit cell growth, does not result in SecY degradation (7). To confirm this result, MG1655 harboring M2-tagged SecY at the chromosomal locus was treated with 100 µg/mL rifamycin. Consistent with the previously reported result (7), treatment with this concentration of rifamycin did not result in a rapid decrease in the concentration of SecY (Figure 4-5). However, this concentration of rifamycin also did not inhibit growth of the cells. Even at 300 µg/mL rifamycin, there was little decrease and SecY concentration, but also no growth inhibition. To test whether SecY concentration would decrease after treatment with a high enough concentration of rifamycin to inhibit cellular growth, the cells were treated with1 mg/mL rifamycin. At this concentration of rifamycin, growth was inhibited, and this was accompanied by a rapid decrease in SecY concentration (Figure 4-5).

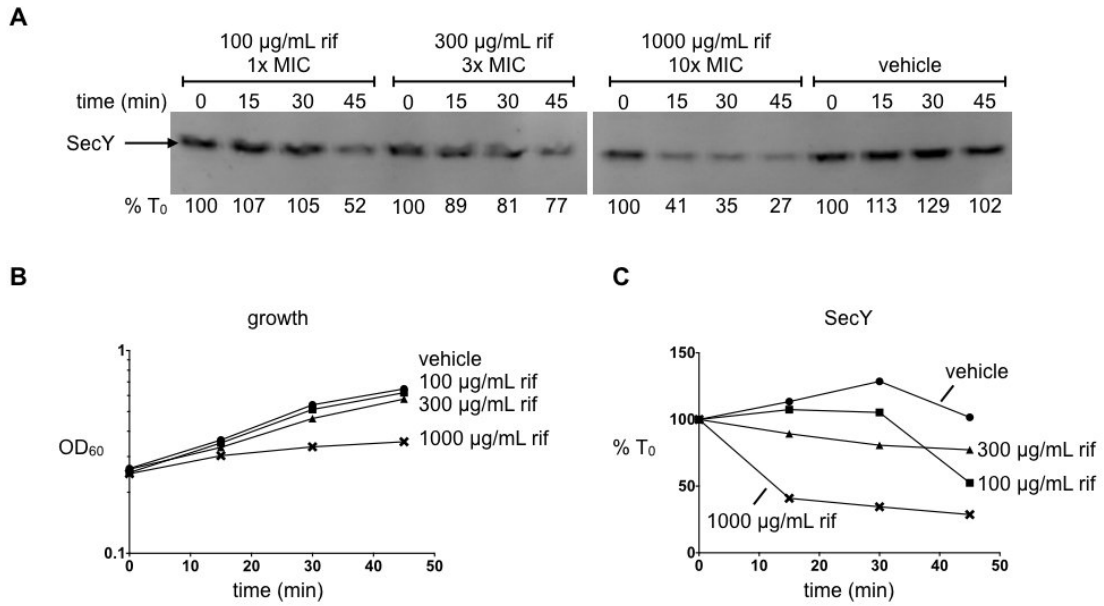

Figure **4-5**: The concentration of SecY is reduced after treatment with rifamycin concentrations that inhibit growth. (A) Western blot showing cellular concentration of SecY after treatment with rifamycin. (B) Growth curve as measured by optical density. (C) Plot of cellular SecY concentrations as a percent of the starting cellular concentration.

Treatment with puromycin results in a dose dependent reduction of SecY

Does the amount of SecY per cell decrease upon treatment with translation and ribosome rescue inhibitors because of channel jamming, or because the cells have stopped growing? If the cellular concentration of SecY is reduced because of channel jamming by the ribosome, then treatment with antibiotics that inhibit growth but do not stall the ribosome should not cause a decrease in SecY concentration. Puromycin specifically hydrolyzes peptidyl-tRNA on the ribosome, which then allows ribosome recycling (16). Therefore, treatment with puromycin should result in cessation of growth, but not channel jamming. To test whether treatment with puromycin resulted in reduced SecY concentration, cells were treated with 1.5, 4.5 or 16 µg/mL puromycin and SecY concentration was monitored by western blot. Treatment with 1.5 µg/mL

puromycin did not result in a reduction of growth, and the cellular concentration of SecY was similar to cells treated with vehicle alone. At 4.5 µg/mL puromycin, a small reduction in SecY concentration was observed compared to the vehicle-treated culture (Figure 4-6). Treatment with 16 µg/mL puromycin resulted in more complete growth inhibition and a rapid reduction of SecY concentration.

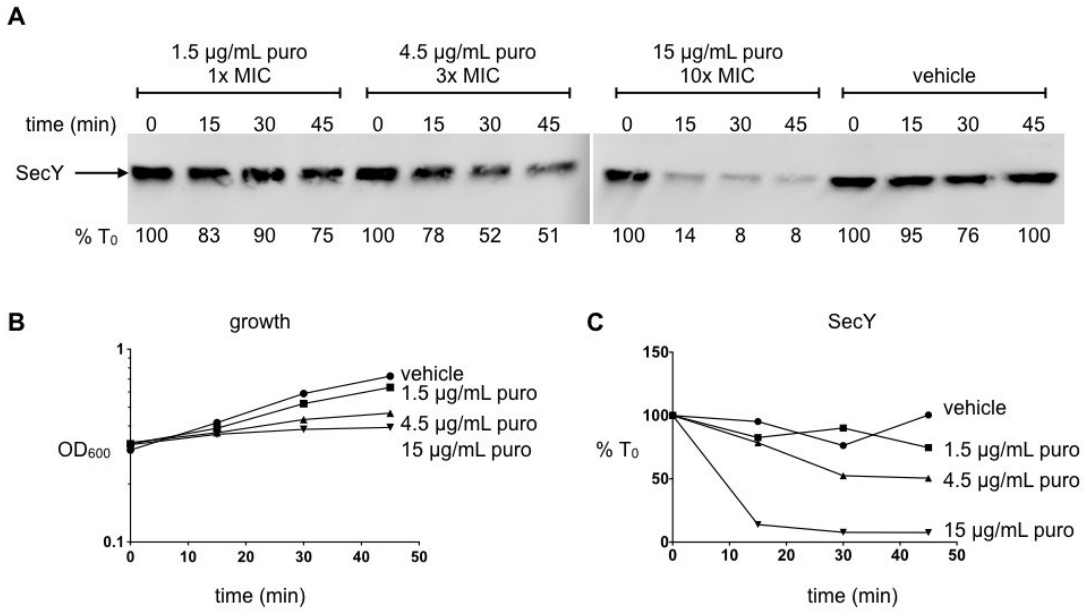

Figure **4-6**: The cellular concentration of SecY is reduced after treatment with puromycin. (A) Western blot showing cellular SecY concentration. (B) Growth of cells monitored by optical density. (C) Concentrations of SecY plotted as a percent of SecY concentration at the start of the experiment.

Deletion of *ftsH* results in increased sensitivity to KKL-35

Destabilization of SecY in response to translation inhibitors has been proposed to contribute to the action of these drugs because SecY is essential (7,10). FtsH has been shown to

be required for SecY degradation in response to chloramphenicol. To test whether an *ftsH* null mutant would have decreased sensitivity to KKL-35, I determined the MIC of this strain in a broth dilution assay. This mutant had slightly increased sensitivity to KKL-35 (Table 4-1). I also tested a strain over-expressing YccA, an FtsH inhibitor, from an IPTG-inducible promoter (7,17). Over-expression of YccA did not affect the MIC of KKL-35.

Table 4-1: Minimum inhibitory concentration of KKL-35 in various strains

Strain	KKL-35 MIC
MG1655Δ*tolC*	0.625 μM
MG1655Δ*tolC ftsH::kan sfhC21*	0.156 μM
MG1655Δ*tolC* p*yccA*	0.625 μM

Materials and Methods

Bacterial Growth

MG1655 was grown at 37 ˚C in LB broth with 0.2% glucose and supplemented with kanamycin (30 µg/mL) where appropriate. DY330 was grown in LB at 30 ˚C.

Construction of MG1655 with M2-tagged SecY at the chromosomal locus

SecY was amplified from the chromosome of MG1655 with primers secY_F and secY_R. The resulting PCR product was used in a second round of PCR with primers secY_F and secY_R2. The resulting product was digested with NcoI and HindIII and ligated into pARCBD-p (18) digested with the same enzymes. Three tandem M2 tags followed by a stop codon were then ligated into an EcoRI site near the 3' end of SecY and the HindIII site by Gibson assembly of a gBlock gene fragment (IDT DNA) to create pSecY3xM2. The fragment included a six residue of linker between the C-terminal of SecY and the M2 tag of the sequence GSAGSA. The Kan cassette of PKD4 was amplified with primers HindIII_PS1_F and HindIII_PS2_R and sub cloned into pJET1.2 (Thermo Fisher Scientific). The cassette was then digested out of pJET1.2 with HindIII ligated into the HindIII site of pSecY3xM2. The resulting plasmid was used as template in a PCR reaction using SecY_F2 and secY_redgam_R. The product generated from this reaction included 431 bases of homology to the 3' end of SecY, the linker and 3xM2 tag, followed by the kan cassette, and 34 bases of homology to the region immediately 3' of SecY on the MG1655 chromosome. This product was transformed into DY330 by electroporation for λ red- mediated recombination as described previously (19). P1 lysate prepared from this strain was used to infect MG1655 or MG1655Δ*tolC* . Transductants were selected on kanamycin at 30 ˚C (Presence of the

kan marker following SecY on the chromosome imparted temperature sensitivity). The presence of flag tagged SecY was confirmed by PCR. The kan marker was then removed by Flp-catalyzed excision (20). After the marker was removed, the resulting strain could be grown at 37 ˚C.

Table 4-2: strains and oligonucleotides

Primer	Sequence
secY_F	ATACCATGGATGGCTAAACAACCGGGATTAGATTTTC
secY_R	TATCTCGAGCGCAGAACCAGAACCACCTCGGCCGTAGCCGTA
secY_R2	TATAAGCTTTTACTTATCGTCGTCATCTTTATAATCCGCAGAAC
HindIII_PS1_F	ATAATAAAGCTTGTGTAGGCTGGAGCTGCTTC
HindIII_PS2_R	TATATATAAGCTTATGGGAATTAGCCATGGTCC
secY_F2	GGAACTGGCTGACAACAATTTCGCTG
secY_redgam_	TTCATTTTTACTCTCCGTAACTTCTCGGGCGACCAAATGGGAA TTAGCCATGGTCC

Strain	Description
DY330	W3110 *ΔlacU169 gal490 λcI857 Δ(cro-bioA)*
MG1655	wild-type *E. coli*
KCK358	MG1655Δ*tolC*
KCK 479	MG1655 with M2 tag appended to C-terminus of SecY at chromosomal
KCK 480	MG1655Δ*tolC* with M2 tag appended to C-terminus of SecY at chromosomal locus
KCK 481	MG1655Δ*tolC ftsH::kan sfhC21*
KCK 482	MG1655Δ*tolC* p*yccA*

Construction of MG1655Δ*tolC ftsH::kan sfhC21*

To construct MG1655Δ*tolC ftsH::kan sfhC21*, the *sfhC21* allele was first transduced into MG1655Δ*tolC* by transduction. *sfhC21* is an allele of *fabZ* that suppresses the lethality of *ftsH* deletion (21) and is linked to *zad-220::*Tn*10* approximately 50,000 bases away. P1 phage lysate was prepared from W3110 *ftsH::kan sfhC21 zad-220::*Tn*10* (gift from Christophe Herman) and used to infect MG1655Δ*tolC.* Transductants were selected on tetracycline. The expected frequency of *sfhC21* with *zad-220::*Tn*10* is about 80%. To ensure that the recipient used in the *ftsH::kan* transduction would have the *sfhC21* allele, several colonies were pooled and then transduced with lysate prepared from W3110 *ftsH::kan sfhC21 zad-220::*Tn*10*, this time with selection on kanamycin to select for *ftsH::kan*. Replacement of *ftsH* with the kanamycin resistance marker was verified by PCR.

Collection and normalization of cultures to measure cellular SecY concentration

Overnight cultures were grown in liquid LB. The next morning 50 mLs of fresh LB was inoculated 1/100 with the overnight culture and allowed to grow at 37 ˚C with shaking for 1.75-2.0 hours to an OD of about 0.16. Glucose was then added to 0.2%. The culture was then split into separate flasks and grown for an additional 15 minutes. At the end of 15 minutes a sample was taken from each culture representing T_0. 1 mL of the culture with the lowest OD was centrifuged at 14,000 RPM for one minute and the supernatant was decanted. The cells were then resuspended in 100 µL of SDS loading buffer. The volume of the remaining cultures to collect was determined by normalization to the OD of that culture. (All subsequent volumes of culture to be collected were normalized to the culture with the lowest OD at T_0.) KKL-35 (dissolved in DMSO) was then added to 0.6 µM (the minimum inhibitory concentration), 1.8 µM, or 6 µM and

aliquots were taken at 15 minute intervals. Cells were resuspended in 100 µL of SDS loading buffer (100 mM Tris [pH 6.8], 3.5% SDS, 20% glycerol, and 4 % beta-mercaptoethanol.)

Western blotting

Samples were resolved on 10% SDS-PAGE gels and transferred to PVDF membrane (Amersham Hybond-P, GE healthcare) at 110 volts for 90 minutes. The membrane was blocked with 3% BSA in TBS-T for 30 minutes. 1µL of anti-M2 antibody (Abcam ab18230) in 10 mL of 3% BSA in TBS-T was applied overnight at room temperature. The membrane was then washed 3 times in TBS-T and then incubated for 2 hours at room temperature with alkaline phosphatase conjugated anti-mouse IgG (Sigma A3562) in 3% BSA. The membrane was then washed 3 times with TBS-T. ECF substrate (GE Healthcare) was then applied to the membrane and signal was visualized with a gel imaging system (Bio-rad). Band intensity was quantified with imageJ.

Discussion and Future Directions

Previous reports have proposed that inhibiting translation causes jamming of the SecY secretion channel which then results in proteolysis of the jammed channel (7). Treatment with KKL-35 also resulted in a rapid decrease in the cellular concentration of SecY, which may indicate that ribosome rescue at the membrane is required to prevent jamming of the translocase. However, treatment with an inhibitor of transcription also resulted in decreased SecY concentrations, when added at high enough concentration to inhibit growth. Transcription and translation are coupled in bacteria, and inhibiting transcription could indirectly result in jamming of SecY. Therefore, I also tested the effect of puromycin on SecY. Puromycin directly hydrolyzes peptidyl-tRNA on the ribosome which should not result in jamming because the peptide is freed from both the ribosome and the tRNA. Cellular SecY concentration was rapidly reduced after treatment with puromycin, suggesting that reduction in SecY concentration that occurs in response to KKL-35 may be a result of growth inhibition rather than channel jamming. However, these possibilities are not mutually exclusive, and it is difficult to determine whether growth inhibition results in reduced SecY, or whether reduced SecY contributes to growth inhibition. Further experiments are necessary to show whether SecY is jammed after treatment with KKL-35 or chloramphenicol.

SecY is universally conserved in bacteria and is essential. Therefore, increased turnover could contribute to the antibacterial action of KKL-35. However, deletion of *ftsH*, which degrades SecY in response to chloramphenicol, resulted in a slight increase in the sensitivity of cells to KKL-35. Degradation of jammed SecY secretion channels is beneficial to cells. Translocases that are engaged with a stalled ribosome are not useful to the cell, and it may be important to clear the cell of these unproductive translocases. However, FtsH is involved in proteolysis of many

additional proteins and also been proposed to have a role as a chaperone, and we can not definitively attribute this effect to SecY stability (21,22).

A further avenue of investigation is to determine whether FtsH is responsible for the reduced SecY concentrations observed after treatment with rifamycin, puromycin, KKL-35 as well as during stationary phase. Proteolysis of SecY by FtsH in response to sudden environmental insult or lack of nutrients could serve as part of a regulatory program to protect the cells. A variety of regulatory networks including mRNA cleavage and ribosome sequestration by hibernation factors are known to inhibit translation during stationary phase (23). A question that remains to be answered is whether translation inhibition during stationary phase results in jammed secretion channels. If so, destruction of SecY could prepare the cells to resume growth after any translational stress by clearing out the unproductive channels.

References

1. Driessen AJM, Nouwen N. Protein translocation across the bacterial cytoplasmic membrane. Annu Rev Biochem. Annual Reviews; 2008;77(1):643–67.

2. Corey RA, Allen WJ, Komar J, Masiulis S, Menzies S, Robson A, et al. Unlocking the Bacterial SecY Translocon. Structure. 2016 Apr 5;24(4):518–27.

3. Hanada M, Nishiyama K, Tokuda H. SecG plays a critical role in protein translocation in the absence of the proton motive force as well as at low temperature. FEBS Lett. 1996 Feb 26;381(1-2):25–8.

4. Voigts-Hoffmann F, Schmitz N, Shen K, Shan S-O, Ataide SF, Ban N. The structural basis of FtsY recruitment and GTPase activation by SRP RNA. Mol Cell. 2013 Dec 12;52(5):643–54.

5. Ménétret J-F, Schaletzky J, Clemons WM, Osborne AR, Skånland SS, Denison C, et al. Ribosome binding of a single copy of the SecY complex: implications for protein translocation. Mol Cell. 2007 Dec 28;28(6):1083–92.

6. Park E, Ménétret J-F, Gumbart JC, Ludtke SJ, Li W, Whynot A, et al. Structure of the SecY channel during initiation of protein translocation. Nature. 2014 Feb 6;506(7486):102–6.

7. van Stelten J, Silva F, Belin D, Silhavy TJ. Effects of antibiotics and a proto-oncogene homolog on destruction of protein translocator SecY. Science. American Association for the Advancement of Science; 2009 Aug 7;325(5941):753–6.

8. Cosma CL, Danese PN, Carlson JH, Silhavy TJ, Snyder WB. Mutational activation of the Cpx signal transduction pathway of Escherichia coli suppresses the toxicity conferred by certain envelope-associated stresses. Molecular Microbiology. 1995 Nov;18(3):491–505.

9. Kihara A, Akiyama Y, Ito K. FtsH is required for proteolytic elimination of uncomplexed forms of SecY, an essential protein translocase subunit. Proc Natl Acad Sci USA. National Academy of Sciences; 1995 May 9;92(10):4532–6.

10. Breukink E. Cell biology. Lethal traffic jam. Science. American Association for the Advancement of Science; 2009 Aug 7;325(5941):684–5.

11. Russell JH, Keiler KC. Subcellular localization of a bacterial regulatory RNA. Proc Natl Acad Sci USA. National Acad Sciences; 2009 Sep 22;106(38):16405–9.

12. Campo N, Tjalsma H, Buist G, Stepniak D, Meijer M, Veenhuis M, et al. Subcellular sites for bacterial protein export. Molecular Microbiology. Blackwell Science Ltd; 2004 Sep;53(6):1583–99.

13. Hayes CS, Keiler KC. Beyond ribosome rescue: tmRNA and co-translational processes. FEBS Lett. 2010 Jan 21;584(2):413–9.

14. Ramadoss NS, Alumasa JN, Cheng L, Wang Y, Li S, Chambers BS, et al. Small molecule inhibitors of trans-translation have broad-spectrum antibiotic activity. Proc Natl Acad Sci USA. National Acad Sciences; 2013 Jun 18;110(25):10282–7.

15. Goralski TDP, Dewan KK, Alumasa JN, Avanzato V, Place DE, Markley RL, et al. Inhibitors of ribosome rescue arrest growth of Francisella tularensis at all stages of intracellular replication. Antimicrob Agents Chemother. American Society for Microbiology; 2016 Mar 7;:AAC.03089–15.

16. Sternberg SH, Fei J, Prywes N, McGrath KA, Gonzalez RL. Translation factors direct intrinsic ribosome dynamics during translation termination and ribosome recycling. Nature Structural & Molecular Biology. Nature Publishing Group; 2009 Aug;16(8):861–8.

17. Kihara A, Akiyama Y, Ito K. Dislocation of membrane proteins in FtsH-mediated proteolysis. EMBO J. EMBO Press; 1999 Jun 1;18(11):2970–81.

18. Scott CP, Abel-Santos E, Wall M, Wahnon DC, Benkovic SJ. Production of cyclic peptides and proteins in vivo. Proc Natl Acad Sci USA. National Academy of Sciences; 1999 Nov 23;96(24):13638–43.

19. Yu D, Ellis HM, Lee EC, Jenkins NA, Copeland NG, Court DL. An efficient recombination system for chromosome engineering in Escherichia coli. Proc Natl Acad Sci USA. National Acad Sciences; 2000 May 23;97(11):5978–83.

20. Cherepanov PP, Wackernagel W. Gene disruption in Escherichia coli: TcR and KmR cassettes with the option of Flp-catalyzed excision of the antibiotic-resistance determinant. Gene. 1995 May 26;158(1):9–14.

21. Ogura T, Inoue K, Tatsuta T, Suzaki T, Karata K, Young K, et al. Balanced biosynthesis of major membrane components through regulated degradation of the committed enzyme of lipid A biosynthesis by the AAA protease FtsH (HflB) in Escherichia coli. Molecular Microbiology. 1999 Feb;31(3):833–44.

22. Herman C, Prakash S, Lu CZ, Matouschek A, Gross CA. Lack of a robust unfoldase activity confers a unique level of substrate specificity to the universal AAA protease FtsH. Mol Cell. 2003 Mar;11(3):659–69.

23. Starosta AL, Lassak J, Jung K, Wilson DN. The bacterial translation stress response. FEMS Microbiol Rev. The Oxford University Press; 2014 Nov;38(6):1172–201.

Chapter 5

The Future of Ribosome Rescue

Introduction

The work I have presented suggests that all bacteria require resolution of nonstop translation complexes for viability. I identified ArfB as a ribosome rescue factor in *Caulobacter crescentus*, and showed that ArfB is required to support viability in the absence of *trans*-translation. All but two bacterial species that are known to be able to survive without *trans*-translation encode a homolog of ArfA or ArfB. I then extended these findings to the mitochondrion, an organelle that arose from a bacterial progenitor, and showed that human cells also require ribosome rescue from nonstop mRNA. Human cells use an ArfB homolog, named ICT1, to rescue nonstop translation complexes, and ICT1 homologs are universally conserved in eukaryotes. Taken together, these results suggest that all cells that require mitochondria for viability may require ribosome rescue activity. The work presented in this dissertation also suggests many additional avenues of investigation and many important questions remain. Why do cells need alternative rescue pathways when *trans*-translation is so efficient? How do the ribosome rescue factors recognize substrate ribosomes? How does inhibiting ribosome rescue result in cell death? These questions will be discussed in detail in this chapter.

Alternative rescue pathways in *Bacillus subtilis* and *Francisella tularensis*

The essentiality of ribosome rescue in a broad and diverse set of bacteria and multiple domains of life strongly suggests that *Bacillus subtilis* and *Francisella tularensis*, two organisms that do not require *trans*-translation, must encode an alternative rescue pathway. In further support of this hypothesis, in March of this year the Craig Venter Institute constructed a minimal bacteria cell with fewer genes than any autonomously replicating cell that has been isolated from nature (1). Among the 473 genes that this minimal cell requires for viability are tmRNA and

SmpB (1). *Trans*-translation is also essential in *Bacillus anthracis* (Alumasa and Keiler, unpublished data), a close relative of *B. subtilis*. The ribosomes of *B. subtilis* are not likely to have diverged so substantially that they are no longer in need of rescue. Efforts to identify alternative rescue pathways in *B. subtilis* (Appendix), and *F. tularensis* are currently ongoing. Identification of these alternative rescue pathways will yield new information about the ribosome and rescue factors that interact with it, and may benefit the design of new antibiotics.

The role of alternative rescue pathways when *trans*-translation is available

Growth conditions under which there is a measurable growth defect imparted by single deletion of either ArfA or ArfB have not yet been identified. Conditions that have been tested include high and low temperatures, minimal media, and treatment with ribosome rescue inhibitors. Broad conservation of ArfB suggests that it imparts a selective advantage, even when *trans*-translation is present. Additionally, ArfA encoded by a nonstop-mRNA in *E. coli*, *Salmonella typhimirium*, *Haemophilus influnzae*, and *Neisseria gonorrhoeae* (2), indicating that *trans*-translation negative regulates ArfA in these species. A regulatory pathway that depends on the inhibition of *trans*-translation suggests that there must be conditions where *trans*-translation is inactivated or limited. This could occur if tmRNA is cleaved, or if the number of nonstop translation complexes exceeds the capacity of *trans*-translation to rescue them.

Trans-translation is the most efficient ribosome rescue pathway because it clears the cell of the nonstop translation complex as well as the aberrant peptide and damaged mRNA. However, there are situations that may necessitate a protein-based rather than an RNA-based solution. For example, stasis in response to nutrient limitation is mediated by RNase toxins. RelE, MazF, HigB, YoeB, and HicA all cleave tmRNA in addition to mRNA (3-6). In contrast, ArfA and ArfB

will not be affected by RNase toxins and can be used until tmRNA levels recover. Compounds that induce amino acid starvation could be used to test whether ArfA or ArfB aid in recovery from toxin stress. Serine hydroxamate (SHX), a seryl-tRNA synthetase inhibitor that starves the cells of serine, induces RNase-type toxin expression (7). tmRNA-mediated degradation of mRNA has been shown to decrease upon addition of SHX (7). To test whether ArfA and ArfB contribute to viability after toxin-mediated stasis, wild-type cells and Δ*arfA* or Δ*arfB* cells could be treated with SHX and then the number of viable cells enumerated. If these alternative rescue pathways are required under these conditions then there should be a quantifiable decrease in recovery from SHX treatment.

Another possibility is that an assault on tmRNA could come from outside the cell. Competing organisms in the environment can inject toxins that either inactivate or overwhelm *trans*-translation. Contact dependent inhibition (CDI) is a strategy used by many bacteria to inhibit growth of neighboring cells, and the targets of these systems are just beginning to be identified (8). Some of these systems rely on toxins that are capable of cleaving RNA, including tRNAs (8). A toxin has not yet been identified that specifically targets tmRNA. However, targeting tmRNA in neighboring bacteria while maintaining an immunity protein (antitoxin) to protect one's own tmRNA would be an effective competitive strategy. Fungi, which don't rely on *trans*-translation, could also encode a nuclease or small molecule inhibitor as part of their repertoire of anti-bacterial defenses. A prediction of this hypothesis is that bacteria that encode alternatives to *trans*-translation would be immune to attack when in the presence of these organisms. Additionally, if we can identify a tmRNA-targeting molecule from microbes in the environment, it would likely have broad-spectrum activity as we have observed for our synthesized compounds. Natural product libraries should be screened for activity against *trans*-translation, since identification of molecules that specifically target *trans*-translation and not ArfA or ArfB could enable the selective targeting of specific pathogens.

Stressful conditions within an animal host may also generate abundant substrates for *trans*-translation. *trans*-translation contributes to the virulence of *Yersinia pestis*, *Salmonella typhimurium*, and *Francisella tularensis* (9-11). Drugs that target ribosome rescue greatly reduce growth of *F. tularensis* in host cells (12). Many pathogenic organisms encode ArfB homologs, including genetically tractable organisms like *Bordetella pertussis* and *Pseudomonas aeruginosa*. A more thorough understanding of which pathogens rely on which rescue systems could greatly aid the development of therapeutics that target specific bacteria.

Recognition of the nonstop translation complex

A universal feature of ribosome rescue by nonstop rescue pathways is a constraint on the length of mRNA downstream of the A site. The activity of ArfA on nonstop ribosomes decreases significantly when the mRNA extends just 3 bases past the A site (13). In chapter 3, I showed that ICT1 and ArfB activity decreases significantly when 14 bases extend past the A site, and activity is near zero when 33 bases extend past the A site. This is consistent with other reports that ArfB activity gradually decreases with increasing mRNA length past the stop codon (13). Kinetic experiments measuring peptidyl transfer onto tmRNA reveal a significant drop from 3 to 6 bases past the A site and near zero activity when 12 bases extend past the A site (14). Experiments in our lab also show a decrease in tagging activity with increasing length of the untranslated mRNA (Haque and Keiler, unpublished data). Finally, Pelota/DOM34 and Hbs1 also have a requirement for length of mRNA past the P site with a significant decrease in peptidyl-tRNA release when 6 bases extend past the A site (15).

Limiting the activity of ribosome rescue factors when there is a significant length of untranslated mRNA downstream of the ribosome is necessary to prevent these pathways from

interfering with translation. How do the rescue factors recognize the actively elongating ribosomes? Crystal structures of both ArfB and SmpB bound to the nonstop ribosome show that they each have a C-terminus that forms a helix occupying the mRNA channel. It was proposed that the C-terminus of SmpB would allow accommodation of only 2 bases past the A site, unless the end of the mRNA can adopt a conformation that differs from the usual path taken through the ribosome (16). Similarly, it was proposed from the structure of *E. coli* ArfB bound to the ribosome that mRNA flexibility would be required to allow 2 or more codons past the A site to be accommodated alongside ArfB. Our data show that ArfB does have significant rescue activity when there are 2 codons past the A site (stop +6), indicating that indeed there is likely to be some movement of the mRNA. What prevents movement of mRNA within the channel when the mRNA extends further? Basic residues of ribosomal proteins uS3, uS4, and uS5 are thought to contact the mRNA as it enters the ribosome and clamp it in place to orient it correctly while it is being decoded (17). We observe some activity of ArfB (chapter 3) and *trans*-translation (Haque and Keiler, unpublished data) when 14 bases extend past the A site. This length of mRNA should make contacts with uS3, uS4 and uS5 and be clamped in place. The rescue activity is low, indicating that we may be observing release activity on transcripts that have some degradation at the 3' end. Further experiments are necessary to show whether (i) the mRNA in our in vitro assay has been either cleaved or degraded and does not extend past the leading edge of the ribosome, (ii) additional contacts between the ribosome and the 3' end of the mRNA work to position the mRNA along its path through the ribosome or (iii) rescue factors recognize some other conformational change in the ribosome independent of a vacant mRNA channel.

Why is rescue essential?

One of the most critical questions that remains is also the most difficult to answer: Why do cells require rescue activity? The simplest answer is that nonstop mRNAs are abundant, and the cell will not be able to make the proteins it needs to survive without rescuing the ribosomes. However, bacterial cells shut down protein synthesis for a variety of reasons without lethal effect. Sporulation, for example, involves a sophisticated series of events that result in a translationally inactive, quiescent state that can last for millions of years. Whereas this type of translation arrest is programmed and may involve protective steps, sudden arrest is also not always lethal. For example, cells in which RNase toxins have cleaved the mRNA in the cell, shutting down protein synthesis, can completely revive (18). A broader goal of the work described in chapter 4 was to determine whether destruction of jammed SecY translocases could be the key to lethality in the absence of ribosome rescue. However, reduced cellular concentrations of SecY in stationary phase suggested that bacteria can recover from secretion stress and that reduced levels of SecY in the cell are not overly deleterious.

Perhaps inactivation of nonstop-ribosome rescue is insurmountable because it is the last resort to solve nearly all problems that can arise during protein synthesis. There is evidence that all kinds of translational stress can result in nonstop translation complexes. Nutrient starvation, prolonged stalling, unprogrammed frame-shifting, mRNA damage, and inactivation of recycling factors have all been shown to result in nonstop translation complexes. Not only does the ribosome initiate translation on truncated mRNA with high frequency, but nonstop mRNA is formed on the ribosome as a fail-safe, so that the ribosome can be rescued from a myriad of problems beyond aberrant mRNA. If the nonstop translation complex is the last resort of the stalled ribosome, inactivating the cell's ability to resolve the nonstop translation complex would be even more catastrophic than we can currently appreciate.

References

1. Hutchison CA, Chuang R-Y, Noskov VN, Assad-Garcia N, Deerinck TJ, Ellisman MH, et al. Design and synthesis of a minimal bacterial genome. Science. American Association for the Advancement of Science; 2016 Mar 25;351(6280):aad6253–3.

2. Garza-Sánchez F, Schaub RE, Janssen BD, Hayes CS. tmRNA regulates synthesis of the ArfA ribosome rescue factor. Molecular Microbiology. Blackwell Publishing Ltd; 2011 Jun;80(5):1204–19.

3. Christensen SK, Gerdes K. RelE toxins from bacteria and Archaea cleave mRNAs on translating ribosomes, which are rescued by tmRNA. Molecular Microbiology. 2003 Jun;48(5):1389–400.

4. Jørgensen MG, Pandey DP, Jaskolska M, Gerdes K. HicA of Escherichia coli defines a novel family of translation-independent mRNA interferases in bacteria and archaea. Journal of Bacteriology. American Society for Microbiology; 2009 Feb;191(4):1191–9.

5. Christensen-Dalsgaard M, Gerdes K. Translation affects YoeB and MazF messenger RNA interferase activities by different mechanisms. Nucleic Acids Research. Oxford University Press; 2008 Nov;36(20):6472–81.

6. Schuessler DL, Cortes T, Fivian-Hughes AS, Lougheed KEA, Harvey E, Buxton RS, et al. Induced ectopic expression of HigB toxin in Mycobacterium tuberculosis results in growth inhibition, reduced abundance of a subset of mRNAs and cleavage of tmRNA. Molecular Microbiology. 2013 Oct;90(1):195–207.

7. Li X, Yagi M, Morita T, Aiba H. Cleavage of mRNAs and role of tmRNA system under amino acid starvation in Escherichia coli. Molecular Microbiology. Blackwell Publishing Ltd; 2008 Apr;68(2):462–73.

8. Aoki SK, Diner EJ, de Roodenbeke CT, Burgess BR, Poole SJ, Braaten BA, et al. A widespread family of polymorphic contact-dependent toxin delivery systems in bacteria. Nature. Nature Publishing Group; 2010 Nov 18;468(7322):439–42.

9. Svetlanov A, Puri N, Mena P, Koller A, Karzai AW. Francisella tularensis tmRNA system mutants are vulnerable to stress, avirulent in mice, and provide effective immune protection. Molecular Microbiology. Blackwell Publishing Ltd; 2012 Jul;85(1):122–41.

10. Okan NA, Mena P, Benach JL, Bliska JB, Karzai AW. The smpB-ssrA mutant of Yersinia pestis functions as a live attenuated vaccine to protect mice against pulmonary plague infection. Infect Immun. American Society for Microbiology; 2010 Mar;78(3):1284–93.

11. Julio SM, Heithoff DM, Mahan MJ. ssrA (tmRNA) plays a role in Salmonella enterica serovar Typhimurium pathogenesis. Journal of Bacteriology. American Society for Microbiology (ASM); 2000 Mar;182(6):1558–63.

12. Goralski TDP, Dewan KK, Alumasa JN, Avanzato V, Place DE, Markley RL, et al. Inhibitors of ribosome rescue arrest growth of Francisella tularensis at all stages of

intracellular replication. Antimicrob Agents Chemother. American Society for Microbiology; 2016 Mar 7;:AAC.03089–15.

13. Shimizu Y. ArfA recruits RF2 into stalled ribosomes. J Mol Biol. 2012 Nov 2;423(4):624–31.

14. Ivanova N, Pavlov MY, Felden B, Ehrenberg M. Ribosome rescue by tmRNA requires truncated mRNAs. J Mol Biol. 2004 Apr 16;338(1):33–41.

15. Pisareva VP, Skabkin MA, Hellen CUT, Pestova TV, Pisarev AV. Dissociation by Pelota, Hbs1 and ABCE1 of mammalian vacant 80S ribosomes and stalled elongation complexes. EMBO J. EMBO Press; 2011 May 4;30(9):1804–17.

16. Neubauer C, Gillet R, Kelley AC, Ramakrishnan V. Decoding in the absence of a codon by tmRNA and SmpB in the ribosome. Science. American Association for the Advancement of Science; 2012 Mar 16;335(6074):1366–9.

17. Takyar S, Hickerson RP, Noller HF. mRNA helicase activity of the ribosome. Cell. 2005 Jan 14;120(1):49–58.

18. Christensen SK, Pedersen K, Hansen FG, Gerdes K. Toxin-antitoxin loci as stress-response-elements: ChpAK/MazF and ChpBK cleave translated RNAs and are counteracted by tmRNA. J Mol Biol. 2003 Sep 26;332(4):809–19.

Appendix

Screen to identify alternative nonstop ribosome rescue mechanism in *Bacillus subtilis*

Special thanks to Chris Johnson of the Grossman lab for advice on using Tn-seq in *B. subtilis*, to Juan Raygoza for guidance on sequencing data analysis, to So Woon "Sophie" Kim for initial screening efforts, and to Chester Price, Dan Kearns, and Kevin Griffith for strains and plasmids.

Introduction

Bacteria rescue ribosomes from nonstop mRNAs using *trans*-translation, ArfA, or ArfB. In *Escherichia coli*, deletion of genes encoding both *trans*-translation and ArfA is synthetically lethal, indicating that ribosome rescue from nonstop mRNAs is essential in *E. coli*. Similarly, deletion of *arfB* in *Caulobacter crescentus* in the absence of *trans*-translation is also lethal. All bacterial species that are currently known to be viable without *trans*-translation encode either ArfA or ArfB homologs, except *Bacillus subtilis* and *Francisella tularensis*. The data presented in this dissertation strongly suggest that these two species must encode an alternative rescue pathway. To identify an alternative rescue pathway in *Bacillus subtilis*, transposon mutagenesis coupled to next generation sequencing (Tn-Seq) was used to identify genes that are essential in cells lacking *trans*-translation. Several candidate genes were identified, including one candidate that encodes a potential RNase III cleavage site 5' of the stop codon, indicating that this candidate could be regulated by *trans*-translation in a similar manner as ArfA.

Results and Discussion

Use of Tn-seq to perform synthetic lethal screen

Several different groups simultaneously developed Tn-seq as a way to detect genes that are essential during a growth condition of interest (1-4). Recently, Tn-seq approaches were comprehensively reviewed by one these pioneering groups (5). Briefly, the bacterial strain of interest is transposon mutagenized and then passaged through a test growth condition, such as an animal host, or temperature stress. Transposon insertion sites are then determined by sequencing. Genes that impart a growth advantage under the test condition will be under-represented in the pool of sequencing reads. This method is particularly well suited for the identification of synthetic lethal gene interactions, where the test condition is the absence of a previously characterized gene product. Tn-seq has several advantages over classical screening methods. Mutant libraries can be generated in a single day and immediately sequenced following exposure to a condition of interest. Currently, massively parallel sequencing of a transposon library is less expensive than the cost of preparing hundreds of agar plates for screening by hand. Tn-seq also allows for rapid detection of the absence of a mutant in a library without time-consuming complementation experiments, or sequential pair-wise deletion of individual genes. Finally, Tn-seq is much more sensitive than traditional methods because genes that impart even a small growth advantage can be detected in a highly complex library.

Transposition of *B. subtilis* wild-type and *ssrA::cat* cells

To identify genes that are essential in the absence of *trans*-translation wild-type and *ssrA::cat*

cells were mutagenized by transducing cells with pMarA, a delivery vector bearing the TnYLB-1 transposon, which encodes a kanamycin resistance cassette flanked by ITR sites. pMarA expresses the *Himar1 C9* transposase under the control of a σ^A promoter. pMarA has a temperature sensitive origin of replication and encodes erythromycin on the plasmid backbone (Figure A-1). After selection on erythromycin at 30 ˚C, individual colonies were grown at 25 ˚C for 10-13 hours, a permissive temperature for pMarA replication. Colonies were then selected on kanamycin at 42 ˚C. To assess the efficiency of transposition, a portion of each culture was plated on kanamycin, erythromycin, or without antibiotics. For wild-type cells, 1.7 ± 0.8 % of the total cell count was kanamycin resistant (number of kan^r colonies/ total colonies x 100). For *ssrA::cat* cells, 1.56 ± 0.16% of colonies were kanamycin resistant. These results are consistent with previous observation of TnYLB-1 transposition frequency (6). Less than 0.003% of colonies recovered from either strain were erythromycin resistant, indicating low retention of delivery vector.

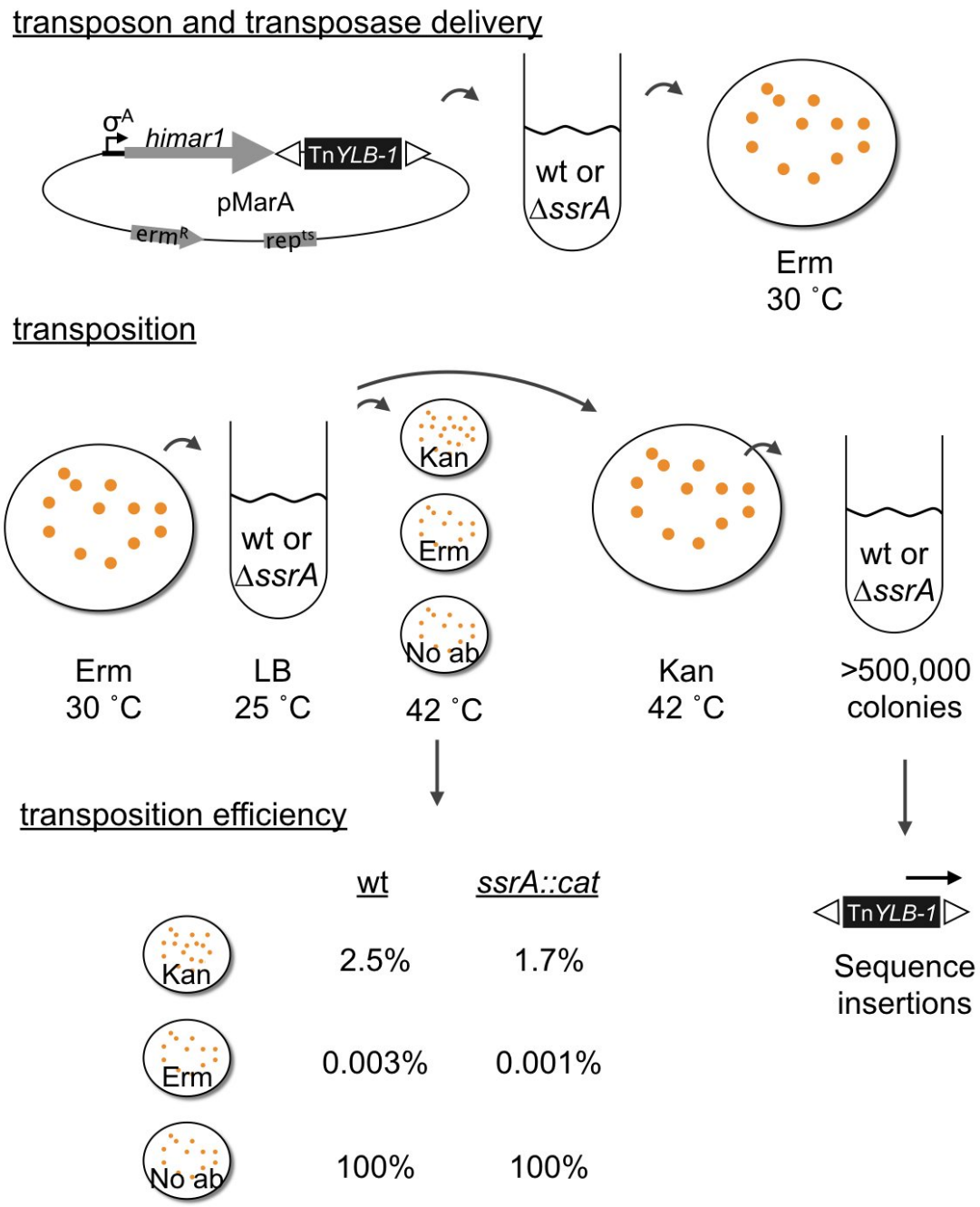

Figure **A-1**: Schematic of transposition of *B. subtilis* to generate transposon libraries. Temperatures at which each step was carried out are indicated. Transposition efficiencies are represented as a percent of colony forming units (CFU) that were kanamycin (Kan) resistant or erythromycin (Erm) resistant out of the total number of colonies that grew on plates without antibiotic (No ab).

Identification of candidate genes

Sequencing of transposon insertions sites yielded more than 9 million reads for the wild-type library and more than 8 million reads for the *ssrA::cat* library. After filtering reads for quality 6,089,919 reads were retained for the wild-type library with 95% alignment rate to the *B. subtilis* 168 genome. After quality filtering the reads obtained from the *ssrA::cat* library 6,835,985 reads were retained and 93% were successfully mapped to the *B. subtilis* genome. There were in total, 39,438 unique insertions were identified in wild-type cells, and 21,314 unique insertions in *ssrA::cat* cells. *Bacillus subtilis* has a genome size of 4.2 Mbp, and thus the wild-type and *ssrA::cat* libraries had on average, a unique insertion every 100 or 200 bases, respectively. Candidate genes were chosen by using a cut off of greater than 5 unique insertions in the wild-type library, and no insertions in the *ssrA::cat* library. These criteria yielded 53 candidates, of which 25 were annotated as having unknown function. Candidate genes are listed in Table A-1.

Table A-1: Candidate genes identified in *B. subtilis* Tn-Seq

gene	annotation	wt insertions	*ssrA::cat* insertions
phrF	phosphatase RapF inhibitor	12	0
yxeJ	hypothetical protein	12	0
ybfA	MarR family transcriptional regulator	10	0
yjzB	hypothetical protein	10	0
ytzE	DeoR family transcriptional regulator	10	0
yuiD	membrane protein	10	0
yaaA	hypothetical protein- putative RNA binding	9	0
yfmL	ATP-dependent RNA helicase YfmL	9	0
xkdH	phage-like element PBSX protein XkdH	9	0

paiA	protease synthase and sporulation negative regulatory protein PAI 1	9	0
ywzA	hypothetical protein	9	0
rpmB	50S ribosomal protein L28	8	0
ymzA	hypothetical protein	8	0
yndN	metallothiol transferase FosB	8	0
ypeQ	hypothetical protein	8	0
kdgT	2-keto-3-deoxygluconate permease	8	0
ureA	urease subunit gamma	8	0
yxlE	negative regulatory protein YxlE	8	0
yxxB	hypothetical protein	8	0
ydhH	hypothetical protein	7	0
yfhH	hypothetical protein	7	0
spxA	regulatory protein spx	7	0
ylmG	membrane protein	7	0
ylqH	hypothetical protein	7	0
yotG	hypothetical protein	7	0
scuA	SCO1 protein homolog	7	0
yppE	hypothetical protein	7	0
ytmP	phosphotransferase YtmP	7	0
trnB-Asp	Asp tRNA	7	0
yurZ	hypothetical protein	7	0
trnSL-Tyr1	Tyr tRNA	6	0
trnSL-Gln2	Gln tRNA	6	0
cotJA	spore coat associated protein CotJA	6	0
yfmA	hypothetical protein	6	0
yhgB	hypothetical protein	6	0
ylaL	hypothetical protein	6	0
ylbN	hypothetical protein	6	0
spoVM	stage V sporulation protein M	6	0
yocN	hypothetical protein	6	0
yoyC	hypothetical protein	6	0
bhlA	holin BhlA	6	0
bdbA	disulfide bond formation protein A	6	0
ypbE	hypothetical protein	6	0
yqjZ	hypothetical protein	6	0
spoIIIAD	stage III sporulation protein AD	6	0
yqaO	hypothetical protein	6	0
yqzO	hypothetical protein	6	0
yqdA	hypothetical protein	6	0

yueG	spore germination protein-like protein YueG	6	0
yukE	hypothetical protein	6	0
yurJ	ABC transporter ATP-binding protein	6	0
ywoH	HTH-type transcriptional regulator YwoH	6	0

The strongest candidate for ribosome rescue is YxeJ

The strongest candidate gene identified is *yxeJ*. This gene encodes a protein of similar size to ArfA that is restricted to *Bacillus subtilis* strains. The *yxeJ* mRNA sequence encodes a significant stem-loop structure 5' of the termination codon containing 21 paired bases (Figure A-2A). RNase III binds and cleaves RNA helices that are approximately 22 bases (7). ARNold terminator predicting software also predicts a terminator 5' of the stop codon (Figure A-2B) (8). Cleavage or termination upstream of the stop codon will result in expression of *yxeJ* from a nonstop message. These data suggest that YxeJ may be negatively regulated by *trans*-translation by a mechanism that is similar to *E. coli* ArfA. YxeJ is restricted to *B. subtilis* and closely related strains and is not found in *Bacillus anthracis*, a closely related species that require *trans*-translation.

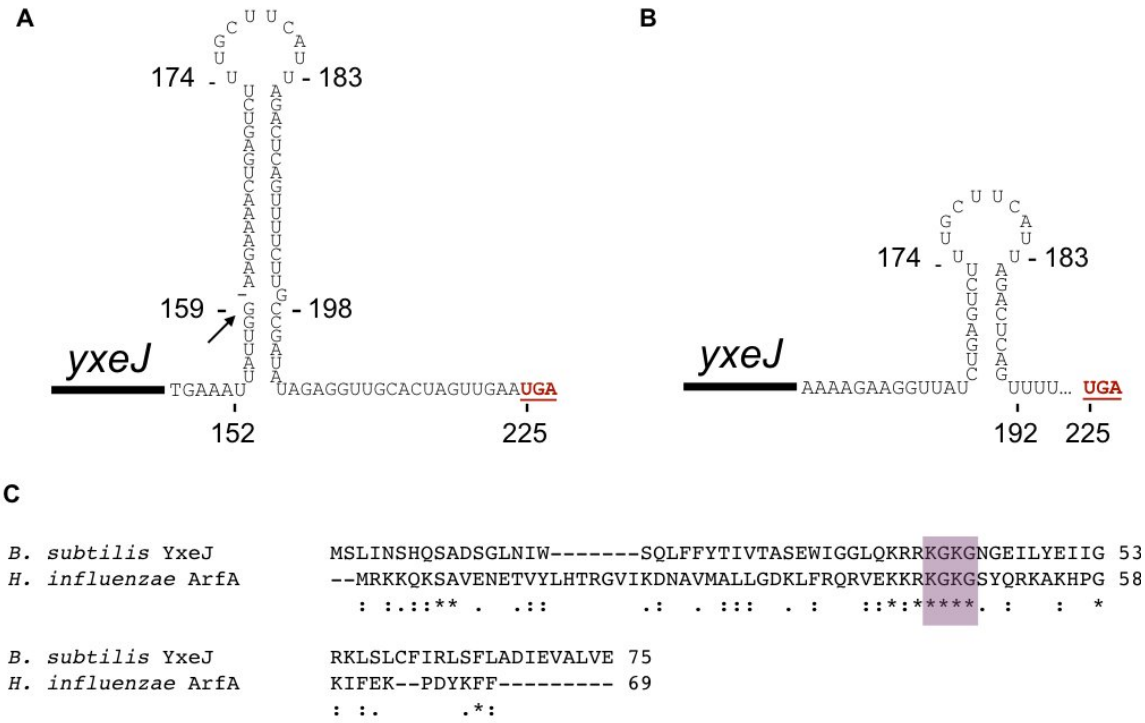

Figure **A-2**: Schematic of stem loop structures in *yxeJ* mRNA and alignment with ArfA. (A) Stem loop structure upstream of the stop codon. Nucleotides are numbered from the start of the gene. The stop codon is colored red. Predicted RNase III cleavage site is indicated by the arrow. (B) Terminator predicted by ARNfold software. (C) Protein sequence alignment between YxeJ and ArfA from *H. influenzae*. Conserved KGKG residues are shaded purple.

YxeJ has less than 13% sequence identity with ArfA of *Haemophilus influenzae*, the ArfA homolog with which it shares the highest identity. However, it does have 28 similar residues out of 75 residues total. Of the ArfA homologs that have been identified in β- and γ- proteobacteria only a single alanine in the N-terminus and five residues (KGKGS) in the middle of the protein are conserved (9). YxeJ encodes KGKG at approximately the same region as ArfA (Figure A-2C). Interestingly, single cysteine substitutions in ArfA at the first lysine of the conserved KGKGS sequence resulted in only slight loss of ArfA:RF2 hydrolysis activity and cysteine substitution at the second lysine or serine residue resulted in no observable loss of ArfA activity.

More extensive mutational analysis to determine ArfA residues that are required for activity may aid the search for additional ArfA homologs.

Future Directions

The next step will be to test purified YxeJ for peptidyl-tRNA hydrolysis activity in vitro. Attempts to express YxeJ in *E. coli* have not been successful. If the stem-loop structure that forms a terminator, or RNase III cleavage site, it should be tagged and degraded in wild-type *E. coli*. Expression was unsuccessful in both wild-type MG1655 and MG1655Δ*ssrA* from both the pBAD and pTrc promoters. *yxeJ* contains three rare arginine codons and expression in a strain containing extra copies the $tRNA^{Arg}$ that decodes these rare codons may increase protein yield. In addition to testing the activity of *yxeJ*, it will be necessary to show whether Δ*yxeJ*Δ*ssrA* is synthetically lethal. If YxeJ has rescue activity but is not required in the absence of *trans*-translation that would suggest that there is a third rescue pathway in *B. subtilis*.

Although this screen yielded promising candidates, the complexity of the transposon libraries generated was not ideal for Tn-Seq. A previous Tn-Seq study in *B. subtilis* capitalized on the fact that this bacterium can uptake linear DNA (10). Transposition of genomic DNA can be performed in vitro and then transformed directly into the cells where it recombines with the chromosome. After transformation, cells can be plated in as little as one hour with selection for those cells that have incorporated the randomly transposed DNA. In vitro transposition is much more efficient than in vivo transposition and bypasses the need for prolonged growth prior to selection, greatly improving library complexity. I was not able to use this approach because *B. subtilis ssrA::cat* is 100 times less competent than the wild-type strain, and so mutagenesis by transformation was not feasible. Recently, our colleague Kevin Griffith infomed us that the

competency defect is due to polar effects on *yvaG*, the gene directly downstream of *ssrA* in *B. subtilis.* Competence is restored in a strain in which *yvaG* has been provided at another locus. Use of this strain will allow us to perform mutagenesis by transformation with in vitro transposed genomic DNA. This strategy will allow us to obtain a library with the saturating insertion density that is ideal for Tn-Seq.

Materials and Methods

Bacterial Growth

Wild-type (BP 1017) *B. subtilis* is a trpC$^+$ derivative of 168 Marburg strain (11). *B. subtilis ssrA::cat* (BP 1020) is a derivative of BP 1017 with the *ssrA* gene disrupted by a cassette encoding chloramphenicol resistance (11,12). Both strains were a kind gift from Chester Price. *B. subtilis* was growth at 37 ˚C with aeration on a roller drum in LB broth unless otherwise specified. Where appropriate, media was supplemented with 100 µg/mL erythromycin, 10 µg/mL kanamycin, 10 µg/mL chloramphenicol and 50µg/mL spectinomycin.

Phage transduction to deliver pMarA

Spp1 lysate was prepared from cells harboring the transposon delivery vector pMarA (13). pMarA bears the transposable element TnYLB-1 consisting of a cassette encoding kanamycin resistance flanked by *Himar1* inverted terminal repeats (ITRs) (6). pMarA also encodes the C9 hyperactive allele of the *Himar1* mariner transposase (14). Recipient wild-type or *ssrA::cat* cells were grown from fresh colonies to stationary phase (about 7 hours) in TY broth (LB supplemented with 10 mM $MgSO_4$ and 100 µM $MnSO_4$). 100 µL of culture was added to serially diluted SPP1 donor phage stock and incubated at 30 ˚C for 30 minutes. Cultures were pelleted and resuspended in 100 µL of TY broth and plated on TY plates with erythromycin and grown at 30 ˚C for 16 hours (wild-type) or 40 hours (*ssrA::cat*).

Table A-1: Strains and oligonucleotides

Name	**Description**	**Reference**
Strains		
KCK483	Wild-type *B. subtilis* 168 trpC^{+}	(11)
KCK484	*B. subtilis* 168 *ssrA::cat*	(11)
KCK485	In-frame deletion of ΔsmpB	(11)
Plasmids		
pMarA	Transposon delivery vector	(6)
p*smpB*	Expresses SmpB under the control of an IPTG inducible promoter	This study
Primers		
himar-seq	AGACCGGGGACTTATCAGCCAACCTGTTA	(15)
SmpB_F	GGAATGGTAGCTAGCGGACAGTTTTTCCTTAG AGAGAGGAGGTTC	This study
SmpB_R	CTAGTATAGCAGGATCCTTAGAAGCCTTTTTGA CTGTCTCTGAACGC	This study
TnYLB_F	CCTGCTAAGGTATATAAGCTGGTGGGAG	This study
TnYLB_R	CAGGCTTGATCCCCAGTAAGTCAA	This study

Transposition

Several isolated colonies transduced with pMarA were picked and grown overnight at 30 ˚C on fresh TY plates containing erythromycin. The next morning, 4 isolated colonies from separate

transduction events were grown in 2 mLs LB broth without antibiotic at 25 ˚C for 10 hours (wild-type) or 13 hours (*ssrA::cat*) to an OD between 1.2-1.5. Cells were plated on LB supplemented with kanamycin and incubated overnight at 42 ˚C, the non-permissive temperature for pMarA replication. Several dilutions were also plated on LB with kanamycin, LB with erythromycin, or LB without antibiotic to determine the transposition frequency. The following morning >500,000 colonies were pooled and resuspended in LB media. Genomic DNA was extracted from 100 µL of pooled colonies using the Wizard genomic DNA preparation kit (Promega). Aliquots of the library were also stored at -20 ˚C in 12% glycerol. The presence of the transposon can be be confirmed with TnYLB_F and TnYLB_R

Transposon insertion site identification

Libraries from wild-type and *ssrA::cat* genomic DNA were prepared and sequenced by Fasteris on a MiSeq instrument. 125 base pair reads were sequenced with the himar-seq primer annealing within the *Himar1* transposon sequence. Reads were trimmed and filtered for quality using Trimomatic (version 0.33) (16). Reads were then aligned to the *B. subtilis* 168 index genome using Bowtie 2 (version 2.2.6) (17). After filtering, 95% of reads from the wild-type library mapped to the *B. subtilis* genome and 93% of reads from the *ssrA::cat* library aligned to the *B. subtilis* genome. Unique insertion sites were enumerated with Tn-seq explorer (18). Insertion sites were visualized with Seq Monk or Geneious software.

References

1. van Opijnen T, Bodi KL, Camilli A. Tn-seq: high-throughput parallel sequencing for fitness and genetic interaction studies in microorganisms. Nat Methods. Nature Publishing Group; 2009 Oct;6(10):767–72.

2. Goodman AL, McNulty NP, Zhao Y, Leip D, Mitra RD, Lozupone CA, et al. Identifying genetic determinants needed to establish a human gut symbiont in its habitat. Cell Host Microbe. 2009 Sep 17;6(3):279–89.

3. Langridge GC, Phan M-D, Turner DJ, Perkins TT, Parts L, Haase J, et al. Simultaneous assay of every Salmonella Typhi gene using one million transposon mutants. Genome Res. Cold Spring Harbor Lab; 2009 Dec;19(12):2308–16.

4. Gawronski JD, Wong SMS, Giannoukos G, Ward DV, Akerley BJ. Tracking insertion mutants within libraries by deep sequencing and a genome-wide screen for Haemophilus genes required in the lung. Proc Natl Acad Sci USA. National Acad Sciences; 2009 Sep 22;106(38):16422–7.

5. van Opijnen T, Camilli A. Transposon insertion sequencing: a new tool for systems-level analysis of microorganisms. Nat Rev Microbiol. Nature Publishing Group; 2013 Jul;11(7):435–42.

6. Le Breton Y, Mohapatra NP, Haldenwang WG. In vivo random mutagenesis of Bacillus subtilis by use of TnYLB-1, a mariner-based transposon. Appl Environ Microbiol. American Society for Microbiology; 2006 Jan;72(1):327–33.

7. Court DL, Gan J, Liang Y-H, Shaw GX, Tropea JE, Costantino N, et al. RNase III: Genetics and function; structure and mechanism. Annu Rev Genet. 2013;47(1):405–31.

8. Gautheret D, Lambert A. Direct RNA motif definition and identification from multiple sequence alignments using secondary structure profiles. J Mol Biol. 2001 Nov 9;313(5):1003–11.

9. Chadani Y, Ono K, Ozawa S-I, Takahashi Y, Takai K, Nanamiya H, et al. Ribosome rescue by Escherichia coli ArfA (YhdL) in the absence of trans-translation system. Molecular Microbiology. Blackwell Publishing Ltd; 2010 Nov;78(4):796–808.

10. Johnson CM, Grossman AD. Identification of host genes that affect acquisition of an integrative and conjugative element in Bacillus subtilis. Molecular Microbiology. 2014 Sep;93(6):1284–301.

11. Shin J-H, Price CW. The SsrA-SmpB ribosome rescue system is important for growth of Bacillus subtilis at low and high temperatures. Journal of Bacteriology. American Society for Microbiology; 2007 May;189(10):3729–37.

12. Muto A, Fujihara A, Ito KI, Matsuno J, Ushida C, Himeno H. Requirement of transfer-messenger RNA for the growth of Bacillus subtilis under stresses. Genes Cells. 2000

Aug;5(8):627–35.

13. Pozsgai ER, Blair KM, Kearns DB. Modified mariner transposons for random inducible-expression insertions and transcriptional reporter fusion insertions in Bacillus subtilis. Appl Environ Microbiol. American Society for Microbiology; 2012 Feb;78(3):778–85.

14. Lampe DJ, Akerley BJ, Rubin EJ, Mekalanos JJ, Robertson HM. Hyperactive transposase mutants of the Himar1 mariner transposon. Proc Natl Acad Sci USA. National Academy of Sciences; 1999 Sep 28;96(20):11428–33.

15. Murray SM, Panis G, Fumeaux C, Viollier PH, Howard M. Computational and genetic reduction of a cell cycle to its simplest, primordial components. Lichten M, editor. PLoS Biol. Public Library of Science; 2013 Dec;11(12):e1001749.

16. Bolger AM, Lohse M, Usadel B. Trimmomatic: a flexible trimmer for Illumina sequence data. Bioinformatics. Oxford University Press; 2014 Aug 1;30(15):2114–20.

17. Langmead B, Trapnell C, Pop M, Salzberg SL. Ultrafast and memory-efficient alignment of short DNA sequences to the human genome. Genome Biol. BioMed Central; 2009;10(3):R25.

18. Solaimanpour S, Sarmiento F, Mrázek J. Tn-seq explorer: a tool for analysis of high-throughput sequencing data of transposon mutant libraries. Antoniewski C, editor. PLoS ONE. Public Library of Science; 2015;10(5):e0126070.

VITA

Heather A. Feaga

Education

2010-2016	Ph.D. Biochemistry, Microbiology and Molecular Biology The Pennsylvania State University, University Park, PA
2007-2010	B.S. Biochemistry and Molecular Biology University of Maryland, Baltimore County, Baltimore, MD

Publications

Feaga HA, Quickel MD, Hankey-Giblin PA, Keiler KC. 2016. Human cells require nonstop ribosome rescue activity in mitochondria. *PLoS Genetics*. 12(3):e1005964.

Hester SE, Goodfield LL, Park J, **Feaga HA**, Ivanov YV, Bendor L, Taylor DL, Harvill ET. 2015. Host specificity of ovine Bordetella parapertussis and the role of complement. *PloS One*. 10(7):e0130964.

Feaga HA, Viollier PH, Keiler KC. 2014 Release of nonstop complexes is essential. *mBio*. 5:e01916-14. doi:10.1128/mBio.01916-14.

Keiler KC, **Feaga HA**. 2014. Resolving nonstop translation complexes is a matter of life or death. *Journal of Bacteriology*. 196:1–9.

Weyrich LS, **Feaga HA**, Park J, Muse SJ, Safi CY, Rolin OY, Young SE, Harvill ET. 2014. Resident microbiota affect Bordetella pertussis infectious dose and host specificity. *The Journal of Infectious Diseases*. 209(6):913-21.

Hester SE, Park J, Goodfield LL, **Feaga HA**, Preston A, Harvill ET. 2013. Horizontally acquired divergent O-antigen contributes to escape from cross-immunity in the classical bordetellae. *BMC Evolutionary Biology*. 13:209.

Feaga HA, Maduka RC, Foster MN, Szalai VA. 2011. Affinity of Cu+ for the copper-binding domain of the amyloid-β peptide of Alzheimer's disease. *Inorganic Chemistry*. 50(5):1614-8.

Awards and Fellowships

2015	Molecular Genetics of Bacteria and Phages Meeting Poster Award
2012-2013	USDA-MFRI Microbial Functional Genomics Fellowship
2010	Homer F. Braddock and Nellie H. and Oscar L. Roberts Fellowship
2010	The Donald Creighton Memorial Scholarship for Award for Outstanding Undergraduate Research
2007-2009	The Academic Achievement Award for Transfers (Scholarship for students transferring from community college with outstanding academic achievement)