RIBOSOME RESCUE IS ESSENTIAL IN BACTERIA AND HUMAN CELLS

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

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).
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 \textit{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 \textit{in vivo} (35).

![Figure 1-2](image)

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) \textit{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.
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).
ArfA is a true backup system for trans-translation in that it is only active when trans-translation activity is not available. The \( \text{arfA} \) mRNA in \( E. \text{coli} \) 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 \( \text{arfA} \) when trans-translation is active results in a tagged ArfA protein that is rapidly degraded. When \( \text{ssrA} \) 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 \( \text{arfA} \) 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 \( \text{arfA} \) suggest that release of ribosomes from nonstop translation complexes is essential in \( E. \text{coli} \) and related species. In \( E. \text{coli} \), deletion of \( \text{arfA} \) and \( \text{ssrA} \) is synthetically lethal (79). In contrast, \( \text{ssrA} \) is essential in \( S. \text{flexneri} \), which does not have \( \text{arfA} \), but \( \text{ssrA} \) can be deleted in \( S. \text{flexneri} \) cells that are engineered to express \( E. \text{coli} \ \text{arfA} \) (64). \( \text{arfA} \) 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 \( \text{arfA} \) makes bioinformatic identification in distantly related bacteria difficult. The presence of \( \text{arfA} \) 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 *arfB* 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.
The discoveries of ArfA and ArfB have important implications for understanding the role of \textit{trans}-translation and the consequences of nonstop translation complexes. With the exception of \textit{B. subtilis} and \textit{F. tularensis}, all species in which \textit{srrA} or \textit{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 \textit{srrA} 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 \textit{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 \textit{trans}-translation.
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.
The results of one high-throughput screen for inhibitors of \textit{trans}-translation have been reported (36,91). Several small molecules identified by HTS inhibit \textit{trans}-translation but not translation \textit{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 $<2 \mu g/ml$ against pathogenic strains of \textit{F. tularensis}, \textit{Y. pestis}, \textit{B. anthracis}, \textit{B. mallei}, and \textit{S. aureus} (91,92), (K. Keiler, unpublished). For KKL-35, growth inhibition of \textit{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 eRF1 and 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(X₃)K(X₀)K(X₂)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.
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Chapter 2

Release of nonstop ribosomes is essential in bacteria

Adapted from:

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 ΔssrA 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 ΔarfA 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 ΔssrA 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 ΔssrA 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 ΔssrA 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 ΔssrA cells. Sequence data were pooled to generate a ratio for each gene (Table 2-1).

Table 2-1 Selected genes identified by Tn-seq

<table>
<thead>
<tr>
<th>gene</th>
<th>insertions in ΔssrA</th>
<th>insertions in wt</th>
<th>log_{10}ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>CC1214</td>
<td>1</td>
<td>564</td>
<td>-2.45</td>
</tr>
<tr>
<td>tRNA^Arg (CCNA R0010)</td>
<td>0</td>
<td>214</td>
<td>-2.33</td>
</tr>
<tr>
<td>rluD</td>
<td>39</td>
<td>2930</td>
<td>-1.87</td>
</tr>
<tr>
<td>lepA</td>
<td>90</td>
<td>5478</td>
<td>-1.78</td>
</tr>
<tr>
<td>efp</td>
<td>3</td>
<td>139</td>
<td>-1.54</td>
</tr>
<tr>
<td>pth</td>
<td>0</td>
<td>24</td>
<td>-1.39</td>
</tr>
<tr>
<td>CC1215</td>
<td>85851</td>
<td>1011</td>
<td>1.93</td>
</tr>
<tr>
<td>smpB</td>
<td>1644</td>
<td>38</td>
<td>1.62</td>
</tr>
</tbody>
</table>

1Number of insertions, normalized to the total number of reads in the larger dataset. 2Ratio 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\(^{\text{Arg}}\)(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 ΔssrA background. Multiple genetic interactions between *pth* and *ssrA* have been reported in *E. coli*, including exacerbation of *pth* mutant phenotypes in ΔssrA cells, so it is not surprising that mutation of *pth* in *C. crescentus* ΔssrA cells results in relatively lower fitness (24). tRNA\(^{\text{Arg}}\)(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\(^{\text{Arg}}\)(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*. 
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 ΔssrA cells does not cause any additional growth defect, resulting in a relatively higher fitness in the ΔssrA 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 ΔssrA cells. Because CC1214 and CC1215 are in an operon, the interactions of both these genes with ssrA were investigated.

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 ΔssrA 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.
**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 ΔssrA cells using the same procedure, suggesting that *CC1214* is essential in the absence of *trans*-translation.

**Table 2-2 Doubling time of mutant strains**

<table>
<thead>
<tr>
<th>strain</th>
<th>doubling time</th>
</tr>
</thead>
<tbody>
<tr>
<td>wt</td>
<td>103 ± 1</td>
</tr>
<tr>
<td>ΔssrA</td>
<td>127 ± 1</td>
</tr>
<tr>
<td><em>CC1214::Ω</em></td>
<td>103 ± 1</td>
</tr>
<tr>
<td><em>CC1215::cat</em> (sense)</td>
<td>101 ± 1</td>
</tr>
<tr>
<td><em>CC1215::cat</em> (antisense)</td>
<td>99 ± 5</td>
</tr>
<tr>
<td>ΔssrA <em>CC1215::cat</em> (sense)</td>
<td>122 ± 1</td>
</tr>
<tr>
<td>ΔssrA <em>CC1215::cat</em> (antisense)</td>
<td>114 ± 4</td>
</tr>
</tbody>
</table>

A co-transduction experiment was used to confirm that loss of *CC1214* is lethal in the ΔssrA 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 ΔssrA cells, none of the 150 kanamycin-resistant colonies were spectinomycin-resistant. When ΔssrA cells containing a plasmid-borne copy of *ssrA* were used as the recipient, the co-transduction frequency was
restored to 51%. Likewise, when ΔssrA 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

<table>
<thead>
<tr>
<th>recipient strain</th>
<th>%CC1214::Ω transductants</th>
<th>% CC1214 transductants</th>
</tr>
</thead>
<tbody>
<tr>
<td>wt</td>
<td>52</td>
<td>48</td>
</tr>
<tr>
<td>ΔssrA</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>ΔssrA pssrA</td>
<td>51</td>
<td>49</td>
</tr>
<tr>
<td>ΔssrA pCC1214</td>
<td>55</td>
<td>45</td>
</tr>
<tr>
<td>ΔssrA pCC1214G30A</td>
<td>0</td>
<td>100</td>
</tr>
</tbody>
</table>

**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 ΔssrA cells. CC1215 could be replaced with the cat gene, encoding chloramphenicol resistance, in both wild-type and ΔssrA 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 ΔssrA 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 ΔssrA cells more fit. Consistent with that hypothesis, CC1215 could not be replaced with an Ω cassette in ΔssrA 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).
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 ΔssrA cells (Table 2-3).
Multicopy expression of CC1214 suppresses the ΔssrA 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 ΔssrA 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.
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 $102 \pm 3$ min, and in ΔssrA cells over-expressing CC1214G30A the doubling time was $175 \pm 11$ 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.
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, *Myxococcus 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, *Desulfbaccula 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

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

<table>
<thead>
<tr>
<th>Strain or Plasmid</th>
<th>Description</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>CB15N</td>
<td>wild-type <em>C. crescentus</em></td>
<td>(32)</td>
</tr>
<tr>
<td>CB15N ΔssrA</td>
<td>In-frame deletion of ssrA</td>
<td>(30)</td>
</tr>
<tr>
<td>CB15N CC1214::Ω</td>
<td>CC1214 replaced by omega cassette</td>
<td>This study</td>
</tr>
<tr>
<td>KCK 426</td>
<td>E. coli BL21 (DE3) pET28CC1214</td>
<td>This study</td>
</tr>
<tr>
<td>KCK 428</td>
<td>CB15N CC1214::Ω with Kan^R marker 22.4 kb downstream</td>
<td>This study</td>
</tr>
<tr>
<td>KCK429</td>
<td>CB15N CC1215 replaced by cat gene in antisense orientation</td>
<td>This study</td>
</tr>
<tr>
<td>KCK430</td>
<td>CB15N CC1215 replaced by cat gene in sense orientation</td>
<td>This study</td>
</tr>
<tr>
<td>KCK431</td>
<td>CB15N ΔssrA CC1215 replaced by cat gene in antisense orientation</td>
<td>This study</td>
</tr>
<tr>
<td>KCK432</td>
<td>CB15N ΔssrA CC1215 replaced by cat gene in sense orientation</td>
<td>This study</td>
</tr>
<tr>
<td>pML81</td>
<td>pJS14-derived plasmid with xylose promoter and NdeI site</td>
<td>M.T. Laub, unpublished</td>
</tr>
<tr>
<td>pML81-His6-CC1214</td>
<td>expresses CC1214 from xylose promoter</td>
<td>This study</td>
</tr>
<tr>
<td>pBBR1-tet</td>
<td>pBBR1 plasmid with <em>tetA</em> cloned into NotI site</td>
<td>gift from Camille Lochte, Pasteur</td>
</tr>
<tr>
<td>Strain or Plasmid</td>
<td>Description</td>
<td>Source</td>
</tr>
<tr>
<td>------------------------</td>
<td>----------------------------------------------------------------------------</td>
<td>-----------------------</td>
</tr>
<tr>
<td>pBBR-PBN</td>
<td>pBBR1-tet derived plasmid with PstI, BamHI, and NdeI cloning sites.</td>
<td>This study</td>
</tr>
<tr>
<td>pCC1214</td>
<td>expresses CC1214 from xylose promoter</td>
<td>This study</td>
</tr>
<tr>
<td>pACC1214G30A</td>
<td>expresses CC1214 active site mutant (GAQ) from xylose promoter</td>
<td>This study</td>
</tr>
<tr>
<td>pSsrA</td>
<td>expresses ssrA from its native promoter</td>
<td>(30)</td>
</tr>
<tr>
<td>pBGS18T(1349674–1350629)</td>
<td>pBGS18T containing targeting fragment 1349674–1350629</td>
<td>(33)</td>
</tr>
<tr>
<td>pET28CC1214</td>
<td>expresses CC1214 from T7 promoter</td>
<td>This study</td>
</tr>
<tr>
<td>pDHFR</td>
<td>expresses DHFR from T7 promoter</td>
<td>New England Biolabs</td>
</tr>
<tr>
<td>pMT425</td>
<td>plasmid with vanillate inducible promoter, <em>cat</em> gene</td>
<td>(34)</td>
</tr>
</tbody>
</table>

**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 mutagenesis. 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

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>1214</td>
<td></td>
</tr>
<tr>
<td>His6F</td>
<td>GGTGATCATATGCAATCATCATCATCATCATCACCCCGCGATGATCGAG</td>
</tr>
<tr>
<td>PstIR</td>
<td>TATCTGCAGTTAGTCGTAATTCACCCGCC</td>
</tr>
<tr>
<td>PstXyl224F</td>
<td>GTCTCGGGCGCGGTCGTCGATGCGAAGGCGCGCCCT</td>
</tr>
<tr>
<td>G30A F2</td>
<td>GCCTCGGCGCGGCGCCACGCTCAACAGACCTCG</td>
</tr>
<tr>
<td>G30A R2</td>
<td>CGAGGTCTTTGTTGACGTCGCTCGGCGCCGGGACCCCGAGG</td>
</tr>
<tr>
<td>1214 UF</td>
<td>ATCCCACTAGTCACCGCCACCGGCTCCACCCG</td>
</tr>
<tr>
<td>1214 UR</td>
<td>TCGATGAATTCGGGCGATCGACGCGACGCGACGCTCAAG</td>
</tr>
<tr>
<td>1214 DF</td>
<td>CGGCTGAAATTCGACGACTAAGACGACGCTCAAG</td>
</tr>
<tr>
<td>1214 DR</td>
<td>TGGACGCTAGCAAGAACGGCAAGGACGACGCTGAAGAA</td>
</tr>
<tr>
<td>1214 Fwd</td>
<td>GGTGATCATATGCAATCATCATCATCATCACCCCGCGATGATCGAG</td>
</tr>
<tr>
<td>1214 Rev</td>
<td>GGGTGAAATTCGACGACTAAGCTTGCTGTC</td>
</tr>
<tr>
<td>1215</td>
<td></td>
</tr>
<tr>
<td>DF</td>
<td>CGCAGGTACTAGCTGGCTGTCGATCGACGCGACGCTGAAGAA</td>
</tr>
<tr>
<td>DR</td>
<td>CGTGAAGGAAAAGCGGATTCAGCCGACGCCGGCC</td>
</tr>
<tr>
<td>UF</td>
<td>CGCAGGTACTAGCTGGCTGTCGATCGACGCGACGCTGAAGAA</td>
</tr>
<tr>
<td>UR</td>
<td>TCGATGAATTCGTCGACGCGACGCGACGCTGAAGAA</td>
</tr>
<tr>
<td>HAF</td>
<td>CGAATTTAATACGACTCAGTATAGG</td>
</tr>
<tr>
<td>T7</td>
<td>AAAACCGTCGTTTGGGATTAGGAGGGTTTGTGATTAGTCCGCCGCTCC</td>
</tr>
<tr>
<td>UTR_DHFR FL</td>
<td>AAACCGTCCCCTCGGTTGAGAGGGTTTGTGATTAGTCCGCCGCTCC</td>
</tr>
<tr>
<td>1214</td>
<td></td>
</tr>
<tr>
<td>ScrnF</td>
<td>CAAGACCTTTTGTGTCGACCAACAGCTGG</td>
</tr>
<tr>
<td>ScrnR</td>
<td>ACCATGACCCTCTATCTGCGAAGATGCTG</td>
</tr>
</tbody>
</table>
Transposon mutagenesis and sequencing (Tn-Seq)

Overnight cultures of wild-type and ΔssrA *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 ΔssrA 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 CB15NCC1214::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. CB15NCC1215::cat was constructed similarly, using primers 1215_UF and 1215_UR to amplify a region ~1.5 kb

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>1215_ScrnF</td>
<td>CTGGAAGCAATATATGCGCCGACAGAC</td>
</tr>
<tr>
<td>1215_ScrnR</td>
<td>CACGTCGAAGCAGCTCGATG</td>
</tr>
<tr>
<td>BBRtetPBN1</td>
<td>CTGCAGGGATCCCATGGCTCCTTGATCGGCGCTTCTTGC</td>
</tr>
<tr>
<td>BBRtetPBN1</td>
<td>CCATGGGGATCCCTGCAGACACCTTCGGGAGCGCCTGA</td>
</tr>
</tbody>
</table>
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 CB15NCC1215::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 ΔssrA. 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 ΔssrA (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$_{600}$ ≈ 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.
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 ΔssrA 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 ΔssrA 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 \textit{trans}-translation? Presumably, bacteria retain \textit{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 \textit{trans}-translation. Alternatively, the simplicity of the single-chain ArfB might have been advantageous during genome reduction.
References


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


Chapter 3

Human cells require nonstop ribosome rescue activity in mitochondria

Adapted from:


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 \textit{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 \textit{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 \textit{C. crescentus} cells that lacked both \textit{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 ΔssrA cells (14), and arfB is essential in C. crescentus ΔssrA 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)
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.

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<tr>
<td>C. crescentus ArfB</td>
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<td>E. coli ArfB</td>
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<tr>
<td>E. coli ArfB</td>
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<td>E. coli ArfB</td>
<td>LAASHHLITSDVIVKAQYESFELNREAATILAVMKIELTEKERPRKMTASSE</td>
</tr>
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</table>

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.
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.
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

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.
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 cotransduction 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 ΔssrA cells that contained ICT1 (Figure 3-3). When the ΔssrA 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 ΔssrA 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 ΔssrA 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 ΔssrA 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 ΔssrA strain expressing ICT1 was measured and compared to control strains. ΔssrA cells expressing ICT1 cells grew at a rate similar to ΔssrA cells expressing ArfB, and significantly faster than ΔssrA 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 ΔssrA cells. Growth of wild-type cells, ΔssrA cells, or ΔssrA 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 blot 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

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**RNAs**

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**Plasmid construction**

To construct pET28ICT1 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 parfB (formerly pCC1214) has been described previously [26]. pICT1 was constructed by digesting pET28ICT1 with Ndel and BamHI and ligating the resulting fragment into parfB digested with the same enzymes. pMCSVICT1 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. pMCSVarfB 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-β-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 PURExpress\(\Delta\RF1,2,3\) 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. PURExpress\(\Delta\RF1,2,3\) 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 \(\mu\)M to inhibit any *trans*-translation activity from tmRNA in the kit components. Samples were precipitated in 20 \(\mu\)l cold acetone, resuspended in loading buffer pH 6.5 (5 mM sodium bisulfite, 50 mM MOPS [morpholinepropanesulfonic acid], 50 mM Tris, 1 \(\mu\)M 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**

\(\Phi CR30\) lysate was prepared from strain KCK428 as described previously (32). The resulting lysate was used to transduce wild-type or \(\Delta ssrA\) cells harboring pICT1, parfB, or empty vector. Overnights of each strain were grown in PYE supplemented with tetracycline and xylose to mid log phase. 25 \(\mu\)l \(\Phi CR30\) 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, $7.5 \times 10^5$ 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 $5 \times 10^4$ cells per well and allowed to adhere for 18 h. Cells were transfected by combining 1 µg pMSCV vector, pMSCV/ICT1, or pMSCVarfB, 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 to 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


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


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 cotranslational 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%.
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.](image-url)
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 with 1 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).
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

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.
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.

![Western blot and optical density graphs showing cellular SecY concentration and growth](image)

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 \textit{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

<table>
<thead>
<tr>
<th>Strain</th>
<th>KKL-35 MIC</th>
</tr>
</thead>
<tbody>
<tr>
<td>MG1655\textit{ΔtolC}</td>
<td>0.625 µM</td>
</tr>
<tr>
<td>MG1655\textit{ΔtolC ftsH::kan sfhC21}</td>
<td>0.156 µM</td>
</tr>
<tr>
<td>MG1655\textit{ΔtolC pyccA}</td>
<td>0.625 µM</td>
</tr>
</tbody>
</table>
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 pARCBDB-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 MG1655ΔtolC. 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

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>secY_F</td>
<td>ATACCATGGATGGCTAAACAAACCCGGGATTAGATTTC</td>
</tr>
<tr>
<td>secY_R</td>
<td>TATCTCGAGCGCAGAGGAACCAGAACCACCTCGGCGTAGCCGTA</td>
</tr>
<tr>
<td>secY_R2</td>
<td>TATAAGCTTTTACTTTATCGTCGTCATCTTTATAATCCGCAGAAC</td>
</tr>
<tr>
<td>HindIII_PS1_F</td>
<td>ATAAATAAGCTTTGTTAGGCTGGAGCTGCTTTC</td>
</tr>
<tr>
<td>HindIII_PS2_R</td>
<td>TATATATAAGGTTATGGAATGAGCATGGTCC</td>
</tr>
<tr>
<td>secY_F2</td>
<td>GGAACTGGCTGACAAACAATTTCCGTG</td>
</tr>
<tr>
<td>secY_redgam</td>
<td>TTCATTTTTACTCTCCGTAACTTTCTCGGCGACCAAATGGGAAATGTAAGCCATGGTC</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Strain</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>DY330</td>
<td>W3110 ΔlacU169 gal490 λcI857 Δ(ero-bioA)</td>
</tr>
<tr>
<td>MG1655</td>
<td>wild-type <em>E. coli</em></td>
</tr>
<tr>
<td>KCK358</td>
<td>MG1655ΔtolC</td>
</tr>
<tr>
<td>KCK 479</td>
<td>MG1655 with M2 tag appended to C-terminus of SecY at chromosomal locus</td>
</tr>
<tr>
<td>KCK 480</td>
<td>MG1655ΔtolC with M2 tag appended to C-terminus of SecY at chromosomal locus</td>
</tr>
<tr>
<td>KCK 481</td>
<td>MG1655ΔtolC ftsH::kan sfhC21</td>
</tr>
<tr>
<td>KCK 482</td>
<td>MG1655ΔtolC pyccA</td>
</tr>
</tbody>
</table>
Construction of MG1655ΔtolC ftsH::kan sfhC21

To construct MG1655ΔtolC ftsH::kan sfhC21, the sfhC21 allele was first transduced into MG1655ΔtolC by transduction. sfhC21 is an allele of fabZ that suppresses the lethality of ftsH deletion (21) and is linked to zad-220::Tn10 approximately 50,000 bases away. P1 phage lysate was prepared from W3110 ftsH::kan sfhC21 zad-220::Tn10 (gift from Christophe Herman) and used to infect MG1655ΔtolC. Transductants were selected on tetracycline. The expected frequency of sfhC21 with zad-220::Tn10 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::Tn10, 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 T₀. 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 T₀.) 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


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 typhimurium*, *Haemophilus influenzae*, 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 \( \Delta \text{arfA} \) or \( \Delta \text{arfB} \) 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 \textit{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 \textit{trans}-translation, could also encode a nuclease or small molecule inhibitor as part of their repertoire of antibacterial defenses. A prediction of this hypothesis is that bacteria that encode alternatives to \textit{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 \textit{trans}-translation, since identification of molecules that specifically target \textit{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


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.


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


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 of 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

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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.
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.

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.
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 MG1655ΔssrA 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*yxeJΔssrA* 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 informed 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

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<td>himar-seq</td>
<td>AGACCGGGGACTTATCAGCCAACCTGTTA</td>
<td>(15)</td>
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<tr>
<td>SmpB_F</td>
<td>GGAATGGTAGCTAGCGGACAGTTTTTCTTGAGAGAGAGGGGTTC</td>
<td>This study</td>
</tr>
<tr>
<td>SmpB_R</td>
<td>CTAGTATAGCGAGCTTTAGGAAGCCTTTTTGACTGTCTCTGAACGC</td>
<td>This study</td>
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<tr>
<td>TnYLB_F</td>
<td>CCTGCTAAGGTATATAAGCTGGTGAGGAG</td>
<td>This study</td>
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<tr>
<td>TnYLB_R</td>
<td>CAGGCTTGATCCCCAGTAAAGTCAA</td>
<td>This study</td>
</tr>
</tbody>
</table>

**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 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


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Education

2010-2016   Ph.D. Biochemistry, Microbiology and Molecular Biology
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Publications


Awards and Fellowships

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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)