SECONDARY GENETIC SCREENING IN BACILLUS SUBTILIS AND BIOCHEMICAL ANALYSIS OF trans-TRANSLATION

A Thesis

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by

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Abstract

Based on strong evidence for the essentiality of ribosome rescue in bacteria, I hypothesize that an alternative rescue factor exists in species where *trans*-translation is dispensable. I also hypothesize that there are conserved biochemical properties that all resolution mechanisms adhere to and may reflect a case of convergent evolution. In Section 2, I present secondary screening in *Bacillus subtilis* as part of an ongoing project to identify an alternative rescue factor using transposon-mutagenesis coupled to deep sequencing (Tn-seq). I extend discussion of the genetic properties of rescue factors to their shared inhibited activity on nonstop ribosomes with increasing 3’-UTRs in Section 3. Taken together, the work in this master’s thesis supports experiments to elucidate character and mechanism of ribosome rescue in bacteria.
# Table of Contents

**List of Figures**

**List of Tables**

## Section 1: Introduction
- Ribosome rescue is essential in eubacteria
- Mechanisms of rescue are diverse
- *B. subtilis* is a Gram-positive model organism that does not require *trans*-translation
- Structural features of rescue factors in nonstop ribosomes
- Shared biochemical properties of ribosome rescue

## Section 2: Secondary screening of a Tn-seq in *trans*-translation null *B. subtilis*
- Abstract
- Introduction
- Materials and methods
- Results
- Discussion

## Section 3: In-vitro *trans*-translation activity is inhibited by extended 3’-UTRs
- Abstract
- Introduction
- Materials and methods
- Results
- Discussion
**List of Figures**

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>2-1</td>
<td>Diagnostic PCR of yxeJ knockout</td>
<td>20</td>
</tr>
<tr>
<td>2-2</td>
<td>Diagnostic PCR of ssrA knockout</td>
<td>21</td>
</tr>
<tr>
<td>2-3</td>
<td>Growth curve of yxeJ and ssrA single and double knockouts</td>
<td>23</td>
</tr>
<tr>
<td>3-1</td>
<td>Hairpin structure of 3′-UTR templates</td>
<td>33</td>
</tr>
<tr>
<td>3-2</td>
<td>In-vitro activity assay of trans-translation</td>
<td>34</td>
</tr>
</tbody>
</table>
## List of Tables

<table>
<thead>
<tr>
<th>Table</th>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>2-1</td>
<td>Strains used in this study</td>
<td>16</td>
</tr>
<tr>
<td>2-2</td>
<td>Primers used in this study</td>
<td>17</td>
</tr>
<tr>
<td>2-3</td>
<td><em>ssrA::cat</em> gDNA transformed into <em>yxef::erm</em></td>
<td>22</td>
</tr>
<tr>
<td>2-4</td>
<td><em>yxef::erm</em> gDNA transformed into <em>ssrA::cat</em></td>
<td>22</td>
</tr>
<tr>
<td>3-1</td>
<td>Primers used for this study</td>
<td>30</td>
</tr>
</tbody>
</table>
Section 1: Introduction

Ribosome rescue is essential in eubacteria

Protein synthesis is an energy-consuming investment in a growing cell, with nearly half the energy of exponential phase *Escherichia coli* devoted to making proteins [1]. Since protein production is the accumulation of many layers of regulation, turnover of translating ribosomes is necessary for homeostasis. In the single compartment cell of bacteria, transcription is coupled with translation. Bacterial ribosomes therefore frequently start translation reactions on mRNAs with no information about their 3’-end. When a transcript is unable to recruit cognate tRNA or release factors, the ribosome remains stuck in an unproductive complex. Stalled mRNAs are cleaved in the A-site and the ribosome translates to the end of a truncated transcript [2]. With no stop codon, the ribosome is ‘nonstop’. Ribosomal rescue is the bacterial mechanism where nonstop ribosomes are released from translation reaction complexes independent of mRNA code. Loss of rescue reduces a cell’s ability to produce protein and buildup of nonstop ribosomes may be lethal [3, 4].

There are currently three ribosome rescue mechanisms known in bacteria: trans-translation, ArfA/RF2, and ArfB. During trans-translation, a large ribonucleoprotein complex made of transfer messenger RNA (tmRNA, encoded by *ssrA*) and its binding partner SmpB (encoded by *smpB*) enter a nonstop ribosome [5, 6]. The alanine-charged tRNA-like domain (TLD) of tmRNA transfers the nascent peptide onto itself, translocates into the P-site powered by EF-G energetics, and loads the open-reading frame (ORF) into the mRNA channel [5, 7]. The ORF is translated canonically as any coding mRNA, appending a hydrophobic degradation tag to the nascent peptide that ends with a stop codon so that the ribosome is
finally freed. The tag is recognized by downstream proteases such as ClpXP and Lon and the defective peptide is cleared [8, 9].

There are also two alternative rescue factors, ArfA and ArfB. These peptide factors promote peptidyl-tRNA hydrolysis in a mechanism distinct from trans-translation and from each other. ArfA is a small peptide that enters the A-site of nonstop ribosomes and recruits Release Factor 2 [10]. RF2 then hydrolyzes the nascent peptide from tRNA and the ribosome is recycled. ArfB is independently catalytic; the factor has the conserved GGQ residues found in Release Factor 1 and 2 to hydrolyze the ester bond in peptidyl-tRNA [11]. Once the peptide is released, the ribosome can be recycled.

Trans-translation has roles in global fitness because it is a full protein quality control system that couples ribosome rescue to proteolysis. It is highly conserved and found in >99% of sequenced eubacteria [12]. Loss of trans-translation is detrimental. It is essential in many species, including Shigella flexneri and Legionella pneumophilia, and has stress-related phenotypes when nonessential, including avirulence in Franciscella tularensis and a cell cycle defect in Caulobacter crescentus [13-16].

In contrast, the environmental or developmental conditions where the alternatives are beneficial remain unknown. Though single deletion appears to have no defect, loss of arfA or arfB is synthetically lethal with trans-translation in E. coli and C. crescentus respectively [17, 18]. Additionally, E. coli ArfA protein is translated from a nonstop transcript that is upregulated when trans-translation is inactive [19]. This is consistent with a physiological role as backup systems in cases where trans-translation is inactive, though conditions where arfA or arfB is advantageous remain unknown. Additionally, the
phylogenetic spread of alternatives is not understood. For example, trans-translation is required in *E. coli* and supported by *arfA*, but is indispensable in the close relative *S. flexneri*, which has an *arfA* homologue that is inactive.

There remain two species, *Bacillus subtilis* and *Franciscella tularensis*, where trans-translation is known to be nonessential and no homologues to *arfA* or *arfB* exist [20]. However, the high turnover of ribosomes, conservation of trans-translation, and presence of backup factors suggest strong selective advantage of ribosome rescue in bacteria. I hypothesize that an alternative rescue factor is present in species where trans-translation is nonessential to resolve nonstop ribosomes.

Mechanisms of rescue are diverse

While trans-translation, ArfA/RF2, and ArfB all provide ribosome rescue, they have distinct mechanisms from each other. The factor diversity may reflect varied physiological need or selection for rescue. For example, under conditions of RNA degradation such as toxin-mediated stasis during nutrient limitation, trans-translation may be vulnerable and the peptide alternatives may be advantageous. Indeed, tmRNA is degraded when the RelE toxin is overexpressed in *E. coli* [21]. Also, developmental states, such as sporulation or reproduction, could have different rescue requirements. For example, tmRNA is degraded during the *C. crescentus* replication cycle [22].

Multiple factors that address the same biological function, such as trans-translation and the alternatives, are found throughout life and hypothesized to be products of convergent evolution. RNA and protein can structurally mimic each other [23], and there are
several instances of RNA and protein factors that synthetically compensate for each other. For example, the signal-recognition protein (SRP) is a universally conserved ribonucleoprotein that binds to newly transcribed peptides and leads them to a membrane for insertion or secretion. In *Saccharomyces cerevisiae*, two alternate pathways exist: GET for tail-anchored proteins and SND for SRP-independent targeting [24]. These protein factors serve as backups for SRP when genetically inactivated. Another striking example is RNase P, the broad name for factors that catalyze tRNA maturation. An ancient RNA-protein complex is found in all three domains of life. There are also protein factors that carry out RNase P activity, such as the single protein PROP [25]. PROP is found widespread in eukaryotes and is interchangeable with the RNA version in *S. cerevisiae* [26]. These two systems of membrane targeting and tRNA maturation represent the paradigm of diverse factors arising to address essential biological functions. From this, it seems likely that bacterial ribosome rescue is a case of convergent molecular evolution, where the strong need to restore unproductive translation reactions drives the development of diverse rescue factors.

*Bacillus subtilis* is a Gram-positive model organism that does not require trans-translation

Of the two species known where trans-translation is nonessential and no homologues to ArfA or ArfB exist, *B. subtilis* is of interest of study as a Gram-positive model organism. In accordance with the Keiler lab’s hypothesis that ribosome rescue is essential in eubacteria, I predict that an alternative rescue factor supports *B. subtilis* in the absence of trans-translation. Gram-positive bacteria represent a phylogenetically divergent branch from Gram-negatives with molecular differences such as a thick peptidoglycan cell wall and a
frequently AT-rich genome [27]. Additionally, bacterial ribosome rescue is an antibiotic target. Small molecules mined for inhibition of trans-translation in \textit{E. coli} have broad-spectrum activity [28]. Further understanding ribosome rescue in distantly related species will elucidate the conservation of trans-translation, the spread of alternatives, and potency as a drug target.

Structural and functional differences in Gram-positive ribosomes may contribute to the mechanistic role of trans-translation and any other rescue factor. For example, the S1 ribosomal protein contributes to translation initiation in \textit{E. coli} and is essential in most Gram-negative bacteria. In contrast, the closest S1 homologue in \textit{B. subtilis} has little known function and is dispensable in most Gram-positive bacteria [29].

The ribosome is also a platform for regulation and is used in species-specific circuits, such as the YidC2 membrane protein in \textit{B. subtilis} [30]. The monocistronic \textit{yidC2} transcript is preceded by \textit{mifM}. The MifM leader peptide stalls the ribosome when translated, so that YidC2 production can be upregulated. However, MifM has no effect on translation rate in \textit{E. coli}, and its stalling in \textit{B. subtilis} is attributed to interactions with point differences in the Gram-positive L22 ribosomal protein. In addition, trans-translation contributes to several regulation mechanisms such as production of the lactose operon repressor, LacI in \textit{E. coli} [31]. At high concentrations in the absence of lactose, LacI binds to its own gene upstream of the stop codon so that transcripts are truncated. The nonstop mRNA and nascent peptide are cleared by trans-translation so accumulation of LacI is prevented. Taken together, these raise the possibility of regulatory circuits that uses trans-translation or ribosome rescue in species-specific manner for \textit{B. subtilis}.
Additionally, *B. subtilis* is a powerful model for developmental states such as natural competency or sporulation. While *trans*-translation is nonessential for viability, genetic deletion of *ssrA* or *smpB* has a growth defect at low and high temperatures, as well as sensitivity to chemical insults such as ethanol and cadmium salts [32]. High throughput phenotyping also suggests *smpB* knockout has delayed entry into sporulation [33, 34]. Further study of ribosome rescue in *B. subtilis* can be used to understand its role in complex physiology.

In this thesis, I present efforts to identify an alternative rescue factor in *B. subtilis* using transposon-mutagenesis coupled to deep sequencing (Tn-seq). Previous work in the Keiler lab used in-vivo transposon mutagenesis to create mutant pools in wild-type and *trans*-translation null *B. subtilis*, and I conduct secondary screening of a top candidate in Section 2.

Structural features of rescue factors in the nonstop ribosome

While the known rescue mechanisms are distinct, the nonstop ribosome is versatile enough to accommodate them. Recent structural data show different contacts of tmRNA/SmpB, ArfA/RF2, and ArfB in the vacant A-site of a nonstop ribosome [35-37]. During typical translation elongation, there are three decoding nucleotides in the A-site that interact with the proper match of codon to anticodon [38]. With unmatched mRNA, A1492 and A1493 are stacked in Helix 44, while G530 has little interaction. Upon cognate anticodon recognition, the three nucleotides flip out to interact with the mRNA-tRNA mini-helix. Canonical termination has different interactions; A1493 stacks with A1913 in Helix 69, and
G530 stacks with the 3rd base in the stop codon [39]. Release factors 1 and 2 (RF1 and RF2) have conserved GGQ residues that catalyze peptidyl-tRNA hydrolysis [40]. When bound to the ribosome the catalytic center is poised in the peptidyl-transferase center (PTC) between the A- and P-site of the polypeptide channel [41].

During trans-translation, SmpB enters the A-site bound to the TLD [35]. The SmpB/TLD is strikingly similar enough to the codon-anticodon structure that EF-G can bind to SmpB and energize translocation [7]. ArfB is a 140-residue peptide with catalytic GGQ motif found in release factors. The N-terminus positions the catalytic residues in the PTC, and the flexible C-terminus samples conformations to enter the mRNA channel [36]. G530 stacks with Arg118, and A1493 contacts Helix 69 [36]. These interactions are similar to release factor binding. Mysteriously, ArfA induces no change in decoding nucleotides when it initially binds to the ribosome [37]. However, once RF2 is recruited, the release factor sandwiches with hydrophobic residues in ArfA so that its GGQ motif is accommodated in the PTC and A1493 stacks within Helix 69. These contacts are similar, though not identical, to stop codon recognition.

**Biochemical properties of ribosome rescue**

Current structural data show rescue factor contacts with the A-site that would be difficult when occupied by mRNA. Factors are recruited independently of template, and the absence of mRNA would be a basis for discrimination between nonstop and translating ribosomes. However, biochemical data has some discrepancy. Transcripts that initiate rescue come from various mechanisms, including stress-related cleavage, damage, or
transcriptional error [20]. Additionally, ribosomes with translation stalled by runs of rare codons or amino acid starvation can be rescued [42-44]. Exonucleases may cleave the A-site mRNA so that rescue factors can be recruited in-vivo, though trans-translation still occurs on stalled transcripts in the absence of exonucleases in-vivo and in-vitro [45]. While truncated mRNAs leave the A-site vacant, stalled transcript substrates cannot exclude an occupied mRNA channel. It may be that there are undiscovered factors that target such ribosomes for rescue or that the A-site mRNA moves to accommodate rescue factors.

Previous work in the Keiler lab shows inhibition of rescue activity on mRNAs with increasing 3′-untranslated regions (UTRs) past a stall site for bacterial ArfB and its mammalian homologue ICT1 [46]. When templates with 6, 14, and 33 nucleotides past a stop codon were incubated in an in-vitro transcription/translation kit where release factors are withhold, the stop codon stalls the ribosome and recruits rescue. With increasing 3′-UTR, ArfB and ICT1 have decreasing rescue activity. Based off this work and supported by other examples of convergent molecular evolution, I hypothesize that the all rescue factors have conserved interactions with nonstop ribosomes during resolution. I continued this study in section 4 of this thesis with an in-vitro trans-translation activity assay using identical mRNA templates with 6, 14, and 33 nucleotides past the stall site, and report the same inhibited activity as 3′-UTRs increase. Taken together, the biochemical data suggest conformations of the nonstop ribosome that are maintained during the various rescue mechanisms.
References

Section 2: Secondary screening of a Tn-seq in \textit{trans}-translation null \textit{B. subtilis}

Abstract

In this section, secondary screening of a candidate low fitness gene identified in a transposon mutagenesis coupled to deep sequencing (Tn-seq) in \textit{B. subtilis} with and without \textit{trans}-translation is presented. This is part of ongoing efforts to identify an alternative rescue factor in \textit{B. subtilis}, which is one of two species where \textit{trans}-translation is known to be nonessential and no homologues to ArfA or ArfB exist. The strong selective advantage of ribosome rescue suggests there is a supporting mechanism to resolve nonstop ribosomes when \textit{trans}-translation is inactivated, and the diversity of rescue factors evade homology searches. Tn-seq is a popular method used to identify novel genes of unknown function based off a selection criteria, and these results show that it is a viable method for measuring mutant fitness in \textit{trans}-translation null \textit{B. subtilis}, though preliminary data is not sufficient to identify an alternative rescue factor.
Introduction

Tn-seq in trans-translation null B. subtilis identified 53 candidate genes

Understanding mechanisms of ribosome rescue in divergent models such as Gram-positive B. subtilis, provides insight into physiological roles of trans-translation and alternatives, including selective advantage and phylogenetic conservation. An alternative rescue factor in B. subtilis is likely to have low or zero fitness in the absence of trans-translation. ArfA and ArfB are synthetically lethal with trans-translation in E. coli and C. crescentus, respectively, consistent with their function as backup rescue mechanisms [1, 2].

Previously in the Keiler lab, transposon-mutagenesis coupled to deep sequencing (Tn-seq) was employed to identify genes that are essential in the absence of trans-translation. Tn-seq is a technique frequently employed in microbes to quantify gene fitness under a selective pressure [3]. Mutant pools are generated through transposon mutagenesis, subjected to a stressor, and surviving mutants are identified and quantified by next-gen sequencing.

The number of transposon insertions in a single gene reflects the frequency of that mutant within the pool, and ratio of insertion after and before selection is a direct measurement of gene fitness. A higher number of inserts indicate mutant tolerance and high fitness. The selection can be anything from abiotic stressors like temperature to genetically engineered background like knockouts. Accurate measurement relies on saturating mutagenesis and a large, complex mutant pool to generate enough reads with respect to a reference measurement before selection. ArfB C. crescentus was successfully discovered in the Keiler lab using Tn-seq comparing genes with no transposon inserts in the ssrA deleted
background to the wild-type background, indicating conditional essentiality in the absence of trans-translation [2].

Previously, a Tn-seq in *B. subtilis* wild-type and ssrA::cat was conducted in the Keiler lab. Mutant pools were generated by in-vivo mutagenesis by temperature-sensitive plasmid pMarA harboring transposase under constitutive promoter and a transposon element marked with a kanamycin resistance cassette. Cultures were grown at 30°C to allow plasmid replication, then raised to 40°C to heat-cure pMarA [4]. Transposed colonies were selected on LB agar supplemented with kanamycin. Mutant pools in wild-type and ssrA::cat backgrounds were collected, extracted for genomic DNA, and sequenced by Illumina MiSeq (Fasteris). Sequencing reads were filtered for quality and mapped to the reference *B. subtilis* Marburg 168 chromosome for location of transposon inserts using the Bowtie pipeline. From the mapped reads, a list of 53 candidate genes was generated with a low ratio of transposon insert in the ssrA::cat to wild-type background, indicating low fitness in the absence of trans-translation.

*yxeJ is the strongest candidate for an alternative rescue factor*

Of the genes identified in the preliminary Tn-seq, *yxeJ* is the strongest candidate for an alternative rescue factor in *B. subtilis*. *YxeJ* is one of two genes with 0 inserts in the ssrA::cat and 12 inserts in wild-type, suggesting essentiality or severe fitness defect. The other low ratio hit is *phrF*, which encodes a phosphatase inhibitor involved in competency though DNA uptake and recombination has little functional overlap with trans-translation. *YxeJ* is a 225 base-pair gene of unknown function that may encode a peptide similar in size
to ArfA. There are no *yxel* homologues in *B. anthracis*, the close relative of *B. subtilis* where *trans*-translation is essential (Alumasa, unpublished data). Additionally, the transcript is predicted to form a cleavage site for RNase III upstream of the stop codon when using RNAfold software. A nonstop transcript could be regulated in a manner similar to *E. coli arfA*.

As part of secondary screening, double knockouts or complements of top hits were constructed to verify and measure fitness defects of genes identified in a Tn-seq. A low number of total hits, insertion hotspots, or large noise signals may give false measurement of gene fitness. In this section, I present construction of the double knockout of *ssrA* and *yxel* in *B. subtilis*, and show that deletion is viable but with a growth defect. These results suggest that Tn-seq is an appropriate method to identify low fitness genes, but preliminary data are not sufficient for discovery of genes conditionally essential with *trans*-translation.

**Materials and methods**

*Strains, growth conditions, and antibiotics*

Strains used and obtained are listed in Table 1. All knockouts were transformed into the wild-type background listed so strains are isogenic. Typical growth conditions were in Lysogeny Broth (LB), in liquid or supplemented with agar, and grown at 37°C. Liquid cultures were grown on roller drum. Agar plates were incubated in oven. Antibiotics were supplemented in media at the following concentrations: chloramphenicol (5 µg/mL), spectinomycin (100 µg/mL), and erythromycin (1 µg/mL).
Table 1: Strains used in this study.

<table>
<thead>
<tr>
<th>Strain name</th>
<th>Notes</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
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<td>Domesticated strain <em>B. subtilis</em> 168 with trpC+</td>
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<tr>
<td>ssrA::cat</td>
<td>Single deletion of tmRNA</td>
<td>[5]</td>
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<tr>
<td>ssrA::cat amyE::ssrA</td>
<td>Single deletion of tmRNA with <em>ssrA</em> complemented under native promoter in <em>amyE</em></td>
<td>[5]</td>
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<tr>
<td>yxeJ::erm</td>
<td>BGSC, BKE collection, locus: 39530</td>
<td>[6]</td>
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</table>

*Genomic DNA purification*

Frozen glycerol stocks of each strain were inoculated into 5 mL of LB and growth overnight. Dense cultures were then collected into a pellet by centrifugation and resuspended in 600 µL of 50 mM EDTA, pH 8.0 and treated with 170 µg/mL lysozyme at 37°C for 5 minutes. Digested cells were pelleted and resuspended in 600 µL of Lysis solution (10 mM Tris-HCl, 2.5 mM EDTA, and 0.5% SDS), heated at 80°C for 5 minutes, then cooled to room temperature. 200 µL of Protein Precipitation Solution (5 M ammonium acetate) was added and mixed vigorously for 20 seconds before cooling on ice for 5-10 minutes. The precipitated slurry was then separated into solid debris and lysate by centrifugation. The supernatant was collected and precipitated with 600 µL of isopropyl alcohol. DNA precipitate was collected by centrifugation and the pellet washed with 70% ethanol and air-dried. The dried DNA was resuspended in 100 µL Elution buffer (10 mM Tris-HCl, pH 8.5) and measured on nanodrop for concentration and purity.

*Competent cell preparation and transformation*

Competent cells of *B. subtilis* were prepared using natural competency. Frozen glycerol stocks were inoculated in LB and grown overnight. Overnights were diluted 1:10 in
freshly prepared MD supplemented with tryptophan (1x PC media, 2% glucose, 20 µg/mL L-tryptophan, 0.1% casamino acids, 3 mM MgSO₄, and 30 µg/mL ferric ammonium sulfate) and grown on roller drum at 37°C to OD 600 greater than 1.0, typically 4 to 8 hours. 400 µL of competent cells were then transferred to new culture tubes and 800 ng of purified genomic DNA pipetted into the mix. The transformation was incubated for 1.5 hours and recovered with warmed LB for 0.5 hours at 37°C. The transformations were plated on LB agar with antibiotics for selection and diluted in plain LB if necessary. Transformation efficiency was measured by the ratio of colony forming units on LB agar with selection divided by nanograms of purified genomic DNA added. Plates were incubated for 24-48 hours, and transformants were selected from single colonies.

**Diagnostic PCR**

Genomic DNA was purified as noted above. 1-2 µL of gDNA was used as template for 20 µL PCRs with Taq DNA polymerase. Primers used are listed in Table 2. HAF primers were designed previously in the Keiler lab.

**Table 2: Primers used in this study.**

<table>
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<th>Primer number</th>
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<th>Sequence</th>
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<tr>
<td>P1</td>
<td>ssrA inside forward</td>
<td>GGGGACGTTACGGATTTCG</td>
<td>This study</td>
</tr>
<tr>
<td>P2</td>
<td>ssrA inside reverse</td>
<td>TATGGAGACGGTGAGGATGCG</td>
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</tr>
<tr>
<td>P3</td>
<td>ssrA outside forward</td>
<td>AAGTTGTCATAGTTCAATAAAGATGAGCG</td>
<td>HAF 3-45</td>
</tr>
<tr>
<td>P4</td>
<td>ssrA outside reverse</td>
<td>CCTATTGGGAGAGTTAAAGACACTTCG</td>
<td>HAF 3-46</td>
</tr>
</tbody>
</table>
Growth curves

Strains were grown in LB overnight and diluted to OD 600 of 0.05. A 96-well plate was seeded with 100 µL of diluted culture and grown for 6 hours in a SpectraMax i3 at 37°C, with medium intensity shake every 5 seconds and an OD 600 read every 20 minutes. 7 replicates and a blank were read for each strain, and doubling time was calculated from the exponential trendline fitted to each growth profile during exponential growth.

Results

Deletion of yxeJ and ssrA in B. subtilis is not synthetically lethal

As a gene with the lowest ratio of transposon inserts in the ssrA::cat to wild-type background, yxeJ was expected to be conditionally essential with trans-translation. To test this, the double knockout of yxeJ and ssrA were constructed, along with yxeJ knockout in an ssrA complemented strain. Genomic DNA was purified from yxeJ::erm and transformed into wild-type, ssrA::cat, and ssrA complement B. subtilis. The complemented strain has the identical deletion of ssrA with the cat cassette, and the ssrA gene expressed from natural promoter from the nonessential amyE locus on the chromosome. Three representative colonies were selected on antibiotic for resistance cassettes marking deletions, extracted for
genomic DNA, and screened for deletion of both \textit{yxeJ} (Figure 1) and \textit{ssrA} (Figure 2) as compared to wild-type and \textit{ssrA::cat}. Diagnostic PCR shows insertion of antibiotic resistant cassettes that mark the deletion of both \textit{yxeJ} and \textit{ssrA} in \textit{B. subtilis}. Two primer sets that anneal inside and outside the gene were used to screen for gene disruption within the gene and in its chromosomal setting. Three representative colonies show that loss of both \textit{yxeJ} and \textit{ssrA} in a strain remains viable.

\textit{Double knockout of \textit{yxeJ} and \textit{ssrA} has a growth defect}

2.5 micrograms of purified genomic DNA from either \textit{ssrA::cat} or \textit{yxeJ::erm} was transformed into WT and \textit{yxeJ::erm} or \textit{ssrA::cat} to make the double deletion of \textit{yxeJ} and \textit{ssrA}. Transformation efficiency was measured by comparing colony forming units on LB agar with selection per microgram of DNA added. Assuming uptake of DNA and recombination are unhindered, when compared to wild-type, \textit{ssrA::cat} (Table 3) and \textit{yxeJ::erm} (Table 4) had less transformation efficiency. The lowered transformation efficiency of double knockout construction suggests fitness defect. This was supported by growth curves of WT and single knockouts of \textit{yxeJ} and \textit{ssrA} to double knockouts (Figure 4). While single deletion of \textit{yxeJ} and \textit{ssrA} slows growth, the double deletion has lower growth rate. Additionally, growth was restored by an \textit{ssrA} complement, suggesting that there are no polar effects of \textit{ssrA} knockouts with respect to growth (Figure 4).
Figure 1: Diagnostic PCR of yxeJ knockout. Two primer sets that anneal outside and within the yxeJ gene were used to show wild-type and insertion of the erythromycin resistance cassette. (a) Diagram showing annealing location of the two primer sets, with (b) table of expected size, and (c) the diagnostic PCR of representative colonies. C1, 2, and 3 are wild-type; C4, 5, and 6 are yxeJ:erm; C7, 8, and 9 are ssrA:cat; and C10, 11, and 12 are yxeJ:erm ssrA:cat.
Figure 2: Diagnostic PCR of ssrA knockout. Two primers sets annealing outside the chromosomal location and within the ssrA gene were used to distinguish wild-type and knockout. (a) Diagram showing where the two primer sets anneal for wild-type ssrA and ssrA::cat, as well as a (b) chart of expected PCR product size. (c) the diagnostic PCR of representative colonies. C1, 2, and 3 are wild-type; C4, 5, and 6 are yxeJ::erm; C7, 8, and 9 are ssrA::cat; and C10, 11, and 12 are yxeJ::erm ssrA::cat.

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<tr>
<td>Inside gene P5 &amp; P6</td>
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<td>Outside gene P7 &amp; P8</td>
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Table 3: *ssrA::cat* gDNA transformed into *yxef::erm*. 2.5 µg of *ssrA::cat* genomic DNA transformed into *yxef::erm* or wild-type *B. subtilis* using natural competency. Transformation efficiency was calculated from the total colony forming units in the transformation divided by the amount of gDNA added in micrograms. The transformation efficiency was compared to wild-type and reported as a percentage.

<table>
<thead>
<tr>
<th>Construction</th>
<th>Transformation efficiency</th>
<th>Compared to wild-type</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>ssrA::cat → yxef::erm</em></td>
<td>1036.8</td>
<td>22.77 %</td>
</tr>
<tr>
<td><em>ssrA::cat → wild-type</em></td>
<td>4550.4</td>
<td>100.00 %</td>
</tr>
</tbody>
</table>

Table 4: *yxef::erm* gDNA transformed into *ssrA::cat*. 2.5 µg of *yxef::erm* genomic DNA transformed into *ssrA::cat* or wild-type *B. subtilis* using natural competency. Transformation efficiency was calculated from the total colony forming units in the transformation divided by the amount of gDNA added in micrograms. The transformation efficiency was compared to wild-type and reported as a percentage.

<table>
<thead>
<tr>
<th>Construction</th>
<th>Transformation efficiency</th>
<th>Compared to wild-type</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>yxef::erm → ssrA::cat</em></td>
<td>126.0</td>
<td>7.06 %</td>
</tr>
<tr>
<td><em>yxef::erm → wild-type</em></td>
<td>1785.6</td>
<td>100.00 %</td>
</tr>
</tbody>
</table>
Figure 3: Growth curve of wild-type, *yxel::erm, ssrA::cat*, double knockouts, and in an *ssrA* complement. (a) Growth in LB media was measured by turbidity in a 96-well plate by a SpectraMax i3 at OD 600, with reads every 20 minutes. Growth profiles show a noticeable growth defect in *yxel::erm ssrA::cat* double deletions that is restored in the *ssrA::cat amyE::ssrA* complement. (b) Doubling times for the different strains shown as an average and standard deviation of 7 replicates. Doubling is given in minutes.

### Doubling Time (mins)

<table>
<thead>
<tr>
<th>Strain</th>
<th>Average</th>
<th>Standard Dev.</th>
</tr>
</thead>
<tbody>
<tr>
<td>wild-type</td>
<td>78.8</td>
<td>2.6</td>
</tr>
<tr>
<td><em>ssrA::cat</em></td>
<td>87.5</td>
<td>6.6</td>
</tr>
<tr>
<td><em>yxel::erm</em></td>
<td>75.8</td>
<td>2.9</td>
</tr>
<tr>
<td>Double KO 1</td>
<td>100.1</td>
<td>6.8</td>
</tr>
<tr>
<td>Double KO 2</td>
<td>150.2</td>
<td>10.1</td>
</tr>
<tr>
<td>Double KO 3</td>
<td>175.9</td>
<td>24.0</td>
</tr>
<tr>
<td><em>ssrA::cat amyE::ssrA</em></td>
<td>70.4</td>
<td>4.4</td>
</tr>
<tr>
<td><em>yxel::erm ssrA::cat amyE::ssrA</em></td>
<td>56.2</td>
<td>3.5</td>
</tr>
</tbody>
</table>
Discussion

*Preliminary Tn-seq may not be sufficiently saturated to identify conditionally essential genes*

The results presented in this section show that \( \text{yxef} \) is not synthetically lethal with \( \text{ssrA} \) in \( B. \text{subtilis} \). The genes \( \text{yxef} \) and \( \text{phrF} \) had the lowest ratio of transposon insertions in the \( \text{ssrA}::\text{cat} \) to wild-type background. If a top hit from the preliminary Tn-seq is not synthetically lethal, it may be that the initial mutant pool is not complex enough as evidenced by secondary screening. Certain genes may be overrepresented in both \( \text{ssrA}::\text{cat} \) and wild-type backgrounds, lowering the signal of gene mutants with low fitness.

However, there is a growth defect of \( \text{yxef}::\text{erm ssrA}::\text{cat} \) double knockout that is more severe than single loss of either gene. The doubling time of double knockout is increased a third of the wild-type doubling time. Single knockout of \( \text{yxef} \) or \( \text{ssrA} \) are slowed by about a tenth compared to wild-type. This suggests that loss of both genes has an added fitness defect. Additionally, \( \text{yxef} \) knockout in an \( \text{ssrA}::\text{cat amyE}::\text{ssrA} \) complement strain has restored doubling time. This suggests that growth defect is independent of chromosomal location of \( \text{ssrA} \) knockout.

Since little biochemical analysis was conducted, the function of \( \text{yxef} \) remains unknown. Genes with low fitness in the absence of trans-translation may be functionally linked to trans-translation. The \( \text{yxef} \) gene could contribute to fitness by relieving nonstop substrates. For example, the Tn-seq conducted in the Keiler lab that identified ArfB as the backup in \( C. \text{crescentus} \) also identified other genes with a low ratio of inserts without trans-translation to wild-type. Such genes included tRNA\(^{\text{Arg}}\)(CCU), which decodes a rare codon, and
Elongation Factor P, which promotes decoding of proline residues. Loss of these factors may create nonstop ribosomes that are difficult to tolerate in the absence of trans-translation.

Though arfA and arfB are synthetically lethal with trans-translation in *E. coli* and *C. crescentus*, it may be that there is another mechanism that relieves nonstop ribosomes in the absence of trans-translation and a backup. Three parallel mechanisms are unlikely, but cannot be ruled out. In that case, a Tn-seq may identify a low fitness but not synthetically lethal gene.

**Further work**

While antibiotic markers appear to have little fitness defect, a prudent control for secondary screening experiments would be to measure the growth defect attributed to the antibiotic resistant markers by integrating in nonessential regions of the chromosome. In this case, the cat cassette that confers chloramphenicol resistance can be controlled by an integration vector, pDG1661, that inserts into the nonessential amyE locus. Likewise, the erm cassette that is selected by erythromycin resistance can be controlled by integration vector pDG1664, which inserts into the nonessential thrC locus. These vectors can be obtained from the Bacillus Genome Stock Center [7]. I have also obtained pDP150, another *erm* marked *thrC* integration vector that can be used as an empty antibiotics control, that is available in my freezer box.

Additionally, an *erm* marked knockout library of all nonessential open-reading frames is available through the BGSC. Further secondary screening can bypass single knockout construction, as I have done. I also have smpB::erm, which is another trans-
translation null *B. subtilis* strain, from the knockout collection. The Gross lab at UCSF also has a kanamycin marked knockout library that is anticipated to become publicly available soon [6]. *Kan* marked gene deletions identified from a Tn-seq in the *ssrA::cat* background can also be screened in the *smpB::erm* knockout.

The data presented here show that the top hit of a preliminary Tn-seq is not saturated enough to identify synthetically lethal genes. To continue the experiment identifying an alternative rescue factor in *B. subtilis*, a modified Tn-seq should be conducted that improves complexity of the mutagenesis.
References

Section 3: In-vitro trans-translation activity is inhibited by extended 3’-UTRs

Abstract

In this section, I expand discussion to the biochemical properties of ribosome rescue that are shared between different factors. Previous work in the Keiler lab has demonstrated similar decreasing rescue activity in bacterial ArfB and its mammalian orthologue ICT1 on in-vitro nonstop ribosomes stalled with mRNAs of increasing 3’-untranslated regions. I continued this study with an in-vitro trans-translation activity assay using identical mRNA templates with 6, 14, and 33 nucleotides past the stall site, and report the same inhibited activity as 3’-UTRs increase. Understanding similar interactions between nonstop ribosomes and rescue factors will elucidate the molecular drive for the essential function. Additionally, insight into the nonstop ribosome enables design of potent inhibitors active against all rescue.
Introduction

Diversity of rescue factors

Previously, I presented secondary screening of *Bacillus subtilis* as part of ongoing efforts to identify an alternative rescue factor. The basis of these experiments is the essential nature of ribosome rescue in bacteria. Rescue resolves nonstop ribosomes, which are translation complexes that are unable to recruit release factors from lack of a stop codon. The known rescue factors, tmRNA/SmpB, ArfA/RF2, and ArfB, have distinct physiology and mechanisms from each other. *Trans*-translation is highly conserved and has detrimental fitness effects when deleted, including essentiality in many species and various stress-related phenotypes in others [1]. ArfA and ArfB serve as backups for *trans*-translation, have no known defect in single deletion, and are synthetically lethal with *ssrA* or *smpB* in *E. coli* and *C. crescentus* respectively [2, 3]. Additionally, the mechanisms are different from each other. *Trans*-translation is mediated by a large ribonucleoprotein, and the backups are peptide factors that promote peptidyl-tRNA hydrolysis [3-5].

*In-vitro rescue activity decreases on mRNAs with increasing 3'-UTR*

Though rescue factors are recruited independent of mRNA code, the length of the template may affect its activity. *Trans*-translation has been shown to have very little activity on mRNAs with over 15 base-pairs past a stall site [7]. Also, previous work in the Keiler lab has shown that bacterial ArfB and its mammalian orthologue ICT1 have decreasing rescue activity on mRNAs with increasing 3'-UTRs [8]. ICT1 is an essential ribosome rescue factor in the mammalian mitochondria with the conserved GGQ residues found in ArfB and release factors [9]. However, it has phylogenetic and structural differences with ArfB, and suggests
that the decreased activity on 3’-UTR templates is conserved for all rescue mechanisms. In this section, I extend this study to trans-translation and show that in-vitro rescue activity is also inhibited by mRNAs with increasing 3’UTRs. Taken together, the biochemical data suggests that there are conserved features of the nonstop ribosome.

**Materials and methods**

**Template production**

Templates to create nonstop ribosomes were made via PCR amplification using the primer sets listed in Table 1. Dihydrofolate reductase (DHFR) was PCR amplified from a control plasmid with a forward primer including the T7 promoter and varied reverse primers that include a stop codon and an untranslated region of varied length. The stop codon, TTA, is underlined and the 3’-UTR is bolded. The template plasmid was PCR amplified with Phusion polymerase, then the amplicon gel purified from 1% agarose gel. The purified DNA served as template for three 50 µL PCRs and their products combined and purified on a silica spin column. Concentration and purity was assessed by measuring A260/280 absorbance using a nanodrop.

**Table 1: Primers used for this study.**

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
<th>Obtained from</th>
</tr>
</thead>
<tbody>
<tr>
<td>DHFR forward</td>
<td>CGAAATTAATACGACTCACTATAGG</td>
<td>HAF 3-72</td>
</tr>
<tr>
<td>DHFR nonstop</td>
<td>AAACCCCTCCGTTTAGAGGGGTGTGTGCTAGATCCGCGCGTCCAGAACATCTCAAAG</td>
<td>HAF 3-71</td>
</tr>
<tr>
<td>DHFR stop+6</td>
<td>AAACCCCTTAGAGTAGAGGTGTGTGCTAGCCCGCGTCCAGAACATCTCAAAG</td>
<td>HAF 3-76</td>
</tr>
<tr>
<td>DHFR stop+14</td>
<td>AAAAACCCTCCGGTTAAGAGGGGTTGCTAGATCCGCGCCGCTCCAGAACATCTCAA</td>
<td>HAF 3-77</td>
</tr>
</tbody>
</table>
In-vitro transcription of tmRNA

The gene encoding *E. coli* tmRNA, *ssrA*, was PCR amplified with Phusion polymerase from pJS14-*ssrA* using primers T7-*ssrA*-forward and T7-*ssrA*-reverse in three 50 µL reactions, combined, purified on silica spin column, then measured by nanodrop for concentration and purity. A 50 µL transcription reaction (100 mM HEPES pH 7.0, 50 mM DTT, 47 mM MgCl₂, 2 mM spermidine, 1 mg/mL BSA, 0.25 µL TIPP, 5 µL RNA polymerase, 20 mM NTP mix with equimolar ATP, UTP, CTP, and GTP, and water to volume) was mixed with 1 µL of *ssrA* template with T7 promoter and terminator. The reaction was incubated at 37°C for 1 hour, then treated with DNase I. The sample was then loaded onto a 5% acrylamide gel with urea and run at 25 miliamps. The gel was imaged using UV light and the tmRNA transcript cut out. The gel piece was suspended in 500 µL diffusion buffer at 37°C overnight, centrifuged, and the supernatant was ethanol precipitated. The pellet was resuspended in RNase-free water and concentration and purity measured by nanodrop.

In-vitro trans-translation reaction

The trans-translation activity in-vitro was measured using the PURExpressΔRF1,2,3 kit from New England Biolabs. The cell-free transcription-translation kit includes T7 RNA polymerase to transcribe DNA template, ribosomes for translation, and is reconstituted without Release Factors 1, 2, and 3. 6 µL reactions were mixed with a microliter of the 4
templates noted above (nonstop, stop+6, stop+14, and stop+33) and 0.35 μL of [\(^{35}\)S]-methionine. The nonstop reaction was supplemented with an antisense oligomer to prevent any tagging from tmRNA/SmpB in the purified ribosomes. 2 μM of tmRNA and SmpB were added to stop+6, stop+14, and stop+33 reactions. Reactions were incubated at 37°C for 1 hour, precipitated with ice-cold acetone, pelleted and resuspended in 15 μL SDS loading buffer (50 mM Tris, 1 μM EDTA, 0.1% SDS, 5% glycerol, 0.01% xylene cyanol, and 0.01% bromophenol blue). 3-5 μL of resuspended reactions were run on 10% SDS-PAGE at 150 V, dried, and exposed to a phosphorimager screen overnight. The screen was then imaged, and the intensity of tagged and untagged bands for each reaction measured by ImageJ.

**Results**

*In-vitro trans-translation activity decreases with increasing 3’-UTRs*

Cell free transcription/translation reactions with reconstituted T7 RNA polymerase and *E. coli* ribosomes (PURExpressΔRF1,2,3, New England Biolabs) were incubated with purified, house-made tmRNA/SmpB and DNA amplicons of DHFR genes without a stop codon (nonstop) and with 6, 14, and 33 nucleotides past a UAA stop codon. The stop codon typically recruits Release Factor 2, but causes stalling in these reactions since release factors are withheld. The 3’-UTRs past the stall site were designed to form hairpin loops to prevent exonuclease digestion (Figure 1). Tagged peptides are indicative of trans-translation rescue. The in-vitro reaction was resolved by gel and rescue activity quantified by the intensity of the heavier molecular weight tagged DHFR to the total tagged and untagged DHFR bands (Figure 2). Compared to nonstop, there is 58%, 22%, and 3% tagging activity for 6, 14, and 33 nucleotides past the stall site.
Figure 1: The predicted structure of 3'-UTR templates that were designed to prevent exonuclease digestion.
Figure 2: In vitro-activity assay of trans-translation. (a) Nonstop template is the positive control, where full trans-translation activity is expected, (b) Stop +6 template, (c) Stop +14 template, and (d) Stop +33 template show have decreasing activity of trans-translation, as represented in bar graph in (e).
Conclusion and discussion

Despite the diversity of rescue factors ArfB, ICT1, and trans-translation, all have activity on reconstituted ribosomes and share decreasing activity on mRNAs with 3’-UTRs of increasing length. Structural data is currently limited to rescue factors in ribosomes with vacant A-sites and make contacts that are difficult to accommodate with mRNA in its typical decoding position [10- 12]. In-vivo, A-site cleavage likely precedes rescue so that stalled transcripts are chewed back leaving the A-site vacant. However, stalling is a substrate for rescue in-vitro in the absence of exonucleases [13]. The biochemical data may mean there are transient interactions of mRNA with a nonstop ribosome. The shared inhibition of rescue by 3’-UTR length from previous studies in the Keiler lab and the work presented here suggest conserved interactions of rescue factors with the nonstop ribosome. Conserved properties of diverse molecular factors may reflect convergent evolution and supports my hypothesis.

The bacterial ribosome has efficient helicase activity that is contributed by multiple structural features, though S3 and S4 of the small ribosomal subunit are essential for this function [14]. mRNA is translated as single-stranded template within the ribosome, so efficient melting of secondary structure is necessary. However, a stall site would prevent translocation and require rescue to resolve the unproductive reaction. Tagged product appears for all templates, suggesting that trans-translation is still recruited though inhibited. Though structured template cannot move within the ribosome, the edge of the mRNA channel may accommodate some secondary formations [15]. The decreasing activity may be from steric prevention of tmRNA/SmpB accommodation in the A-site. A follow up experiment would be to change the hairpin loop of the 3’-UTR with bases that do not form
secondary structure. If rescue activity increases, it may mean that structural features of the mRNA template contribute to rescue factor recruitment.

Alternatively, exonucleases may degrade the hairpin loop that remains exposed outside the ribosome [16]. A larger, more complex 3’-UTR may require further digestion before rescue can occur. The reaction conditions used here are an endpoint assay that quantifies rescue at a single timepoint. Reaction conditions are controlled to prevent exonucleases, but contamination cannot be excluded. This can be tested by spiking the reaction with exonucleases. For example, if the in-vitro transcription/translation kit is supplemented with a known concentration of exonuclease and rescue activity is maintained, this will suggest that template degradation does not contribute to the rescue factor recruitment.

While rescue factors differ from each other in physiology and mechanism, all address the essential function of nonstop ribosome resolution. Indeed, the ribosome is versatile and accommodates diverse trans factors [17]. However, there may be molecular features of the nonstop state that drives convergent evolution of rescue factors as evidenced by shared biochemical activity such as the inhibition by 3’-UTR presented here. The most conspicuous characteristic of nonstop ribosomes is recruitment of factors independent of mRNA code. While truncated mRNAs leave the A-site vacant, a stalled template may have to move to accommodate rescue factors. There are also nuanced molecular signal transductions within a macromolecular machine like the ribosome [18], and there may be some that indicate a nonstop state. Further studies include structural and kinetic studies of rescue factor recruitment to a substrate ribosome, including competition of different rescue factors. Since
Ribosome rescue has been shown to be a potent drug target [6], further understanding the shared properties between all nonstop ribosomes undergoing rescue enables cognizant design of promising therapeutics.
References