Evaluating *Francisella tularensis* translation *in vitro*

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Thesis Proposal

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A. STATEMENT OF THE PROBLEM

Francisella tularensis is a Gram-negative, facultative, intracellular bacterium which causes the disease tularemia. Previous research has demonstrated that *F. tularensis* ribosomes are heterogeneous and that a specific ribosomal protein, bS21-2, regulates translation. To study the regulation of translation by ribosomes in *Francisella tularensis*, I will use an *in vitro* system. I aim to develop a reporter construct for use in an *in vitro* translation assay that is sensitive and easy to modify and to reproducibly purify active ribosomes from *F. tularensis* for use in this assay.

B. JUSTIFICATION FOR THE STUDY

Francisella tularensis is a Gram-negative, facultative, intracellular bacterium. *F. tularensis* causes the disease tularemia, which can be transmitted by rabbits, small rodents, or arthropods (Sjöstedt, 2007). This disease manifests at the onset with flu-like symptoms and can develop into several forms, of which the ulceroglandular form is the most common and the pneumonic form can be fatal (Degabriel et al., 2023). Because a relatively small exposure to the bacterium can cause an infection and it can be easily dispersed through the air, it has been classified as a potential bioweapon (Oyston et al., 2004). The Ramsey lab uses *F. tularensis* subspecies *holarctica*, specifically the live vaccine strain (LVS), as a model organism, because it is not infectious to humans but retains its virulence in animal models. The Ramsey lab studies post-transcriptional regulation of virulence genes in *F. tularensis*, specifically how changes in ribosome composition impact virulence gene expression.

Key virulence genes in *F. tularensis* are located within the Francisella Pathogenicity Island (FPI), a region of the genome that codes for the type 6 secretion system (T6SS). The T6SS is a protein complex necessary for bacterial intracellular survival and growth within host cells. This cluster of genes is tightly regulated at the level of transcription. Specifically, transcription increases during the stringent stress response when the alarmone (p)ppGpp binds to transcription factors MglA and SspA associated with RNA polymerase and recruits another transcription factor, PigR. This increases transcription from promoters that contain short motifs called PigR response elements (PREs), including those found on the FPI (Degabriel et al., 2023). Recent work has demonstrated that the expression of FPI virulence genes is further controlled by regulating the translation of the mRNA into proteins by a specific ribosomal protein, bS21-2 (Trautmann & Ramsey, 2022).

Most bacteria encode one bS21 homolog or none at all. In the ribosome, bS21 is located in the small subunit near the anti-Shine Dalgarno sequence and the channel where mRNA exits the ribosome. Its location suggests that bS21 may interact directly with the 5’ untranslated region (5’ UTR) of mRNA molecules during the initiation phase of translation. Several studies have implicated bS21 in translation initiation by *E. coli* ribosomes (van Duin & Wijnands, 1981; Chang & Craven, 1977).

F. tularensis is unusual in that it has three homologs of bS21. The Ramsey lab found that *F. tularensis* ribosomes can incorporate any one of the three bS21 homologs and that the second homolog, bS21-2, is the most abundant when cells are grown in the laboratory (Trautmann & Ramsey, 2022). Additionally, in the absence of bS21-2, the abundance of certain proteins, including T6SS proteins, decreased, yet mRNA abundance remained the same

(Trautmann & Ramsey, 2022). This suggests that, when bS21-2 is absent, ribosomes cannot translate these genes as efficiently. We also found that the ability of cells to replicate within macrophage decreased when bS21-2 was absent (Trautmann & Ramsey, 2022). These data lead to the development of a model in which bS21-2 allows for more efficient translation of virulence genes.

In support of a model in which bS21-2 controls translation initiation, it was found that the presence of bS21-2 can affect the translation of mRNAs with specific 5' UTR sequences and that altering the 5' UTR sequences can lead to a loss of this regulatory effect (Trautmann et al., 2023). In particular, the Ramsey lab studied these effects *in vivo* using a reporter assay to examine how the presence of bS21-2 alters translation of particular mRNAs in *F. tularensis*. These reporters consisted of a control promoter, the promoter for the gene *tul4* (*tul4* is not regulated by bS21-2 (Trautmann & Ramsey, 2022)), driving the transcription of an mRNA containing the 5´ UTR of a control gene or gene of interest fused to a green fluorescent protein (GFP) coding sequence. GFP production was used as a measure of translation efficiency for each mRNA. The 5' UTRs tested in this reporter assay were selected based on a proteomics analysis in which the absence of bS21-2 led to a downregulation in protein production from the selected genes (Trautmann & Ramsey, 2022). These genes included the FPI genes *pdpA* and *iglA*. When bS21-2 was absent, reporter output decreased for the selected genes but did not decrease for *tul4* (Figure 1; Trautmann & Ramsey, 2022). Since changes in reporter output correlate with the amount of protein in cells with and without bS21-2, these data indicate that the 5´ UTR is sufficient to influence translation by bS21-2 *in vivo*. Additionally, because 5´ UTRs are generally

important for initiation, these results strongly suggest that bS21-2 regulates translation during initiation.

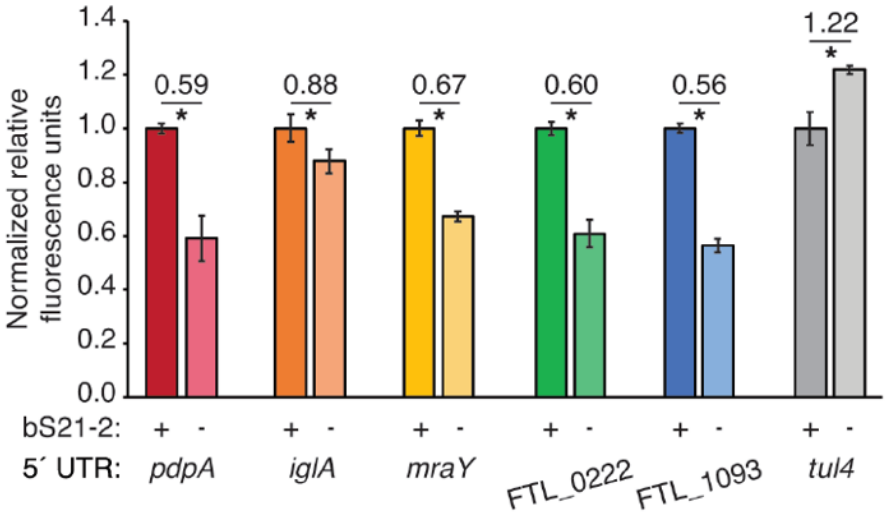

Figure 1. 5´ UTRs are sufficient to lead to bS21-2-mediated changes in translation. Relative fluorescence for indicated GFP translational fusion reporters in cells with (+; WT) or without (−; Δ*rpsU2*) bS21-2. The *tul4* 5´ UTR serves as a control. Figure and legend are from Trautmann et al., 2023.

After determining that the 5´ UTR is sufficient to allow bS21-2-mediated changes in translation, the Ramsey lab further investigated 5´ UTR sequences to identify specific elements that lead to regulation. In most bacterial 5´ UTRs, a particular sequence called the Shine-Dalgarno (SD) pairs with the anti-Shine-Dalgarno (aSD) sequence in the 16S rRNA of the 30S ribosomal subunit. This pairing is not strictly necessary for translation initiation but increases its efficiency. Structures of *E. coli* ribosomes reveal that bS21 likely directly contacts the aSD sequence (Kaledhonkar et al., 2019), implicating the SD-aSD pairing as a potential candidate for regulation by bS21-2 in *F. tularensis*. Upon testing translation efficiency of mRNAs with different SD sequences, the Ramsey lab found that 5´ UTRs with ideal SDs lost responsiveness

to bS21-2 (Figure 2A, Trautmann et al., 2023), suggesting that bS21-2 interacts with the 5´ UTR in certain ways, but that the presence of a perfect SD can override these effects so that they are not as significant. In other genes, however, imperfect SD sequences do not automatically lead to regulation, so the search for other regulatory elements continues.

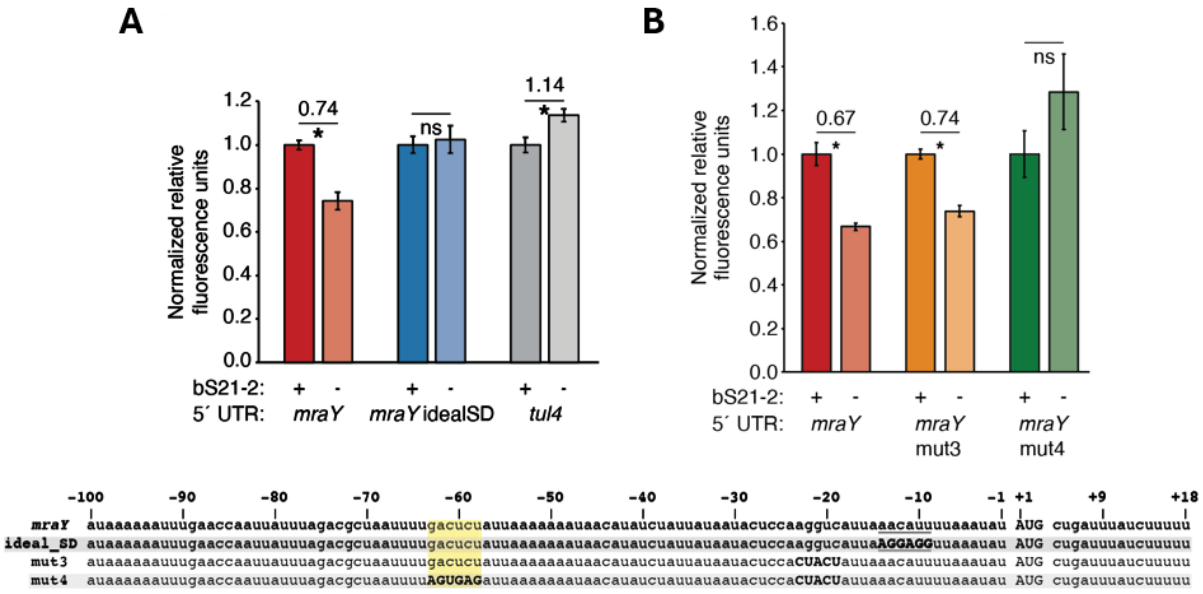

Figure 2. **bS21-2-mediated translation of *mraY* depends on a weak Shine-Dalgarno and a specific six nucleotide sequence.** Charts show relative fluorescence for indicated *gfp* translation fusion reporters in cells with (+; WT) or without (−; Δ*rpsU2*) bS21-2. (A) Introduction of an ideal SD in the *mraY* leader leads to loss of bS21-2 responsiveness. (B) The nucleotides between −58 and −63 in the *mraY* 5´ UTR, GACUCU, are essential for responsiveness to bS21-2. (A–B) Lines above bars indicate comparisons, values above line indicate ratio of reporter activity in cells lacking bS21-2 to wild-type cells. Error bars represent 1 SD. *P < 0.05 by t-test. ns = not significant. Experiments were repeated at least twice in biological triplicate, and data from a representative experiment are shown. Figure and legend are from Trautmann et al., 2023.

To find elements necessary for regulation by bS21-2, the Ramsey lab investigated the 5´ UTR of *mraY*. Hannah Trautmann tested the contribution of two motifs (AAAAUAAA and UUAUUUA) that are enriched in the 5´ UTR of genes regulated by bS21-2 as well as the predicted secondary structure of 5´ UTRs, but these did not contribute to regulation by bS21-2.

Through further testing and modification of the *mraY* 5´ UTR, she identified a 6-nucleotide motif in the *mraY* 5´ UTR GACUCU (Figure 2B, Trautmann et al., 2023) that is necessary for regulation by bS21-2.

These studies have allowed significant progress in understanding how bS21-2 controls translation of mRNAs with specific 5' UTRs, but we would like to go further and address several other questions. In particular, we would like to determine if these results can be recapitulated *in vitro*. Once a working *in vitro* system has been established, we can also test whether the GACUCU motif is sufficient for regulation by bS21-2 and whether there are other sequence motifs that confer regulation. By developing an *in vitro* assay that allows us to examine translation efficiency, we can build upon previous experimental data and develop a more comprehensive model of regulation by bS21-2.

The goal of my thesis project is to develop an *in vitro* translation assay for *F. tularensis*. In developing this assay, I will use the PURExpress® Δ Ribosome Kit from New England Biolabs which contains all the factors necessary for transcription and translation except ribosomes and permits the user to translate any protein as a reporter. When the kit components are combined with a DNA template and purified ribosomes, transcription and translation of a DNA-encoded reporter will occur, resulting in a signal that can be measured to assess translation efficiency. To use this kit for our studies, we must clone a plasmid template and purify active ribosomes from *F. tularensis*.

Preliminary work towards developing an *in vitro* translation assay performed by Hannah Trautmann used *F. tularensis* ribosomes and the *tul4* 5' UTR – GFP reporter. While the reaction

with *E. coli* ribosomes (provided by the kit) yielded signal in the assay, showing the reaction was properly set up, the ribosomes purified from *F. tularensis* did not translate detectable amounts of product (Figure 3). These results suggested to us that we should try to find a more sensitive reporter and that we should ensure that the ribosomes we are purifying are sufficiently active.

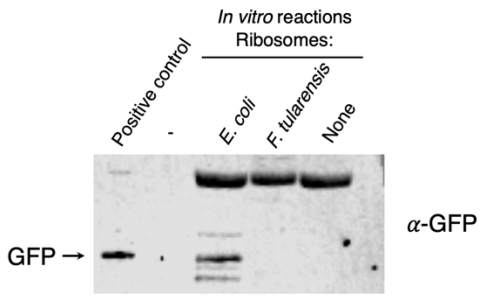

Figure 3. Ribosomes purified from *F. tularensis* exhibited poor activity in initial *in vitro* assays. *In vitro* translation assays were performed using a DNA template specifying an mRNA with the *tul4* 5' UTR fused to the GFP gene (pKR107). Reactions contained either control ribosome from the kit (from *E. coli*), ribosomes purified from *F. tularensis* LVS, or no ribosomes (as indicated at the top of the image). No fluorescence was detected from the reaction containing *F. tularensis* ribosomes, so samples were analyzed by immunoblot using an antibody to GFP. The positive control lane contains cell lysate from *F. tularensis* expressing GFP.

C. AIMS

Specific Aim #1: Develop a reporter construct for use in an *in vitro* translation assay that is sensitive and easy to modify.

To address my first aim, I have assessed multiple reporters to see which provides the highest signal. These reporters include the fluorescent proteins GFP, LanYFP, and iLov, and the luciferase protein nLuc. To do this, I cloned the genes encoding these reporters into an existing plasmid that allowed for transcription by the *in vitro* translation kit. After measuring translation efficiency using these reporter constructs, I chose nLuc because of its broad dynamic range and high signal strength in comparison to the other reporters (Figure 4).

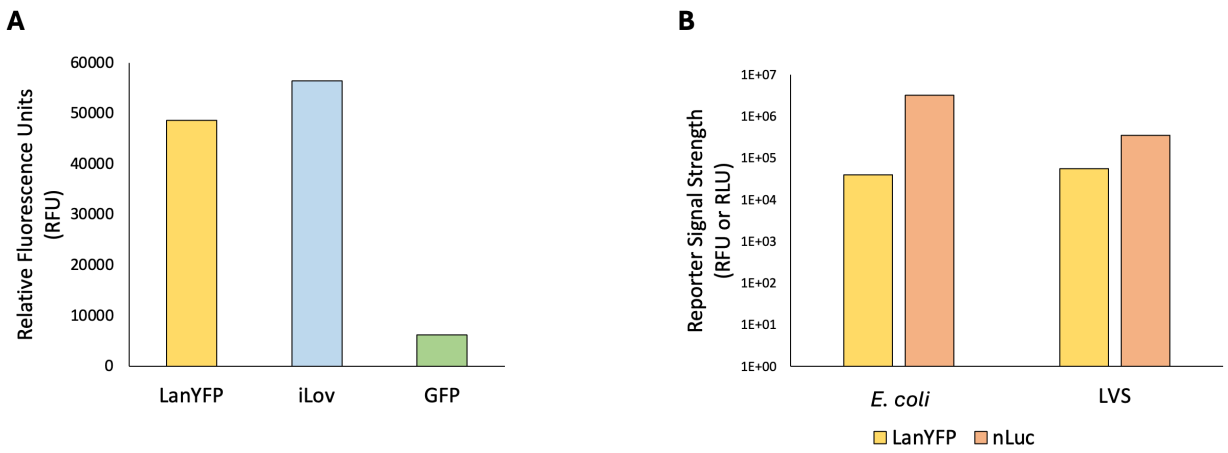

Figure 4. Comparison of reporters demonstrates that nLuc is the most sensitive reporter tested. (A) Comparison of signal from fluorescent reporters detected in cells. Chart shows relative fluorescence for indicated translation fusion reporters (LanYFP, iLov, and GFP). (B) Comparison of LanYFP fluorescence and nLuc luminescence. Reporter signal strength is displayed in relative fluorescence units or relative luminescence units for indicated translation fusion reporters (LanYFP and nLuc) produced from *in vitro* assays using *E. coli* or LVS ribosomes respectively (note log scale). *In vitro* translation assays were performed using the pKR204 reporter template, which specifies a *pdpA* 5' UTR – nLuc reporter fusion and a *tul4* 5' UTR – LanYFP reporter fusion.

To easily switch out UTR sequences in front of the *nLuc* coding sequence using standard cloning techniques, I modified an existing plasmid containing *nLuc* fused to the 5' UTR for *pdpA*. This has provided us with a reporter construct that is sensitive and easy to modify for use in our *in vitro* translation assay.

Specific Aim #2: Reproducibly purify active ribosomes from *E. coli* and *F. tularensis*.

To isolate active ribosomes, I have been using the sucrose cushion purification method. I first harvested ribosomes from *E. coli* cells grown in LB medium and was able to achieve a yield of 1.5-5 nmol from 1 L of culture (about 1.75g of cells) with a concentration of 10-30 pmol/µL, which is substantially higher than the 2.7 pmol/µL concentration required for the *in vitro* translation kit. Next, I demonstrated that the *E. coli* ribosomes were active using the *in vitro*

assay and replicated these results using ribosomes from multiple purification attempts, showing that purification of *E. coli* ribosomes using the sucrose cushion method is reproducible (Figure 5).

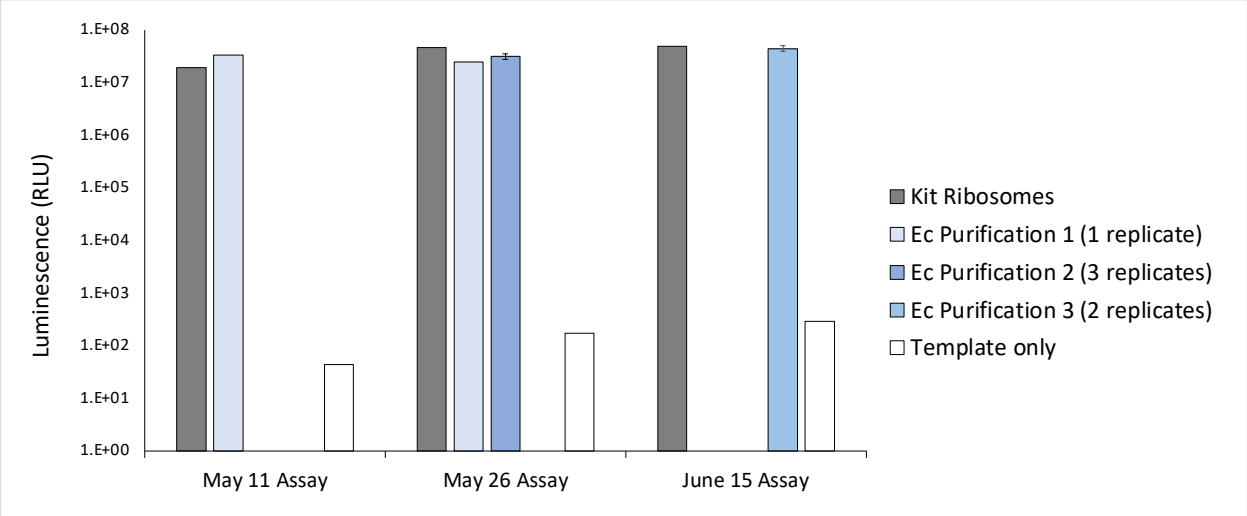

Figure 5. Relative luminescence values for nLuc after translation by purified *E. coli* ribosomes. Results of *in vitro* translation assays using the *pdpA* 5´ UTR-*nLuc* reporter (pKR144) showing signal strength for multiple *E. coli* (Ec) ribosome purifications tested on different days. Ribosomes were purified using the sucrose cushion method. Kit ribosomes are also from *E. coli* and were supplied with the NEB assay kit.

After validating the sucrose cushion method using *E. coli* ribosomes, I purified ribosomes harvested from *F. tularensis* LVS cells grown in brain-heart infusion (BHI) media. I tested reproducibility by measuring nLuc luminescence signal from the same sample of sucrose cushion purified ribosomes on different days and signals from different sucrose cushion purifications on the same day (Figure 6). I observed reproducible translation with *E. coli* ribosomes, but a significant degree of variability in translation using *F. tularensis* ribosomes, including replicate to replicate variability.

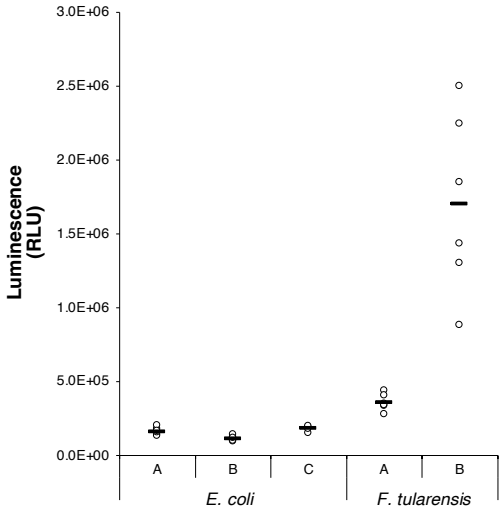

Figure 6. Assessing translation by ribosomes from *E. coli* and *F. tularensis*. Results of an *in vitro* translation assay of the *tul4* 5´ UTR-*nLuc* reporter (pKR214) by indicated ribosomes. Letters indicate biological replicate purifications of ribosomes, each circle represents a technical replicate.

Given the variability in translation by ribosomes isolated from *F. tularensis*, I took several approaches. First, I made changes to the *in vitro* assay protocol to standardize experiments, including resuspending ribosome pellets overnight to ensure homogeneity, taking the average of several concentration reads to increase accuracy, storing single-use aliquots to reduce freeze/thaw cycles, and using a master mix in the assay to reduce pipetting error. I also analyzed ribosome samples on sucrose gradients, revealing that purified *E. coli* ribosomes exhibit prominent 70S peaks along with additional peaks that we suspect correspond to polysomes (Figure 7). When analyzing *F. tularensis* ribosomes, we found distinct 70S peaks as well as peaks that might correspond to 50S subunits and 100S ribosome dimers. These 100S dimers form when two 70S ribosomes interact with hibernation factors and undergo conformational changes and are usually accompanied by downregulation of translation (Prossliner et al., 2018). Since it appears that we are isolating both 70S and 100S ribosome particles together, we expect that there is a subset of purified ribosomes within each sample that are inactive, potentially leading to the observed variability. My current experiments focus on isolating homogenous 70S ribosomes to further ensure consistency between *in vitro* assays.

This will provide us with active ribosomes from *F. tularensis* for use in our *in vitro* translation assay.

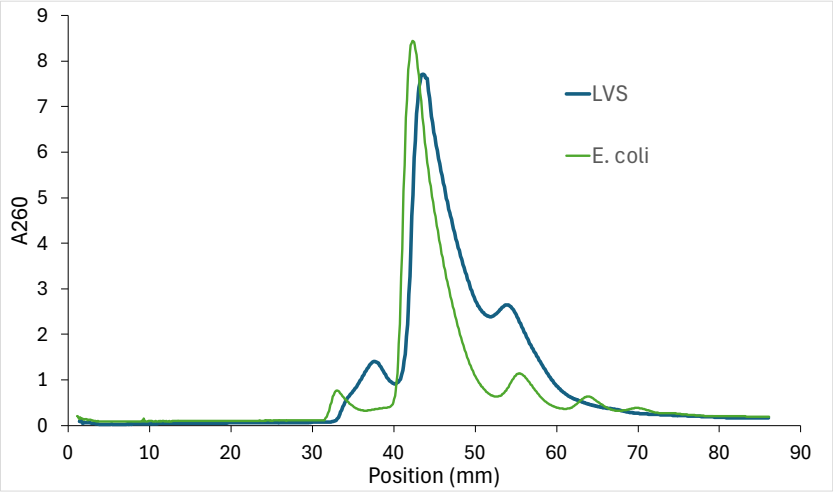

Figure 7. Sucrose gradient sedimentation profiles of purified ribosomes from indicated species. Sucrose gradient profiles of either *E. coli* (green) or *F. tularensis* LVS (blue) ribosomes. Ribosomes were purified using the sucrose cushion method.

Ongoing experiments are focused on producing homogenous, reproducibly active 70S ribosomes from *F. tularensis*. There seems to be a correlation between the amount of ribosomes present in a sample and how much aggregation is observed, so I will seek to determine the optimal amount of ribosomes to use in an assay that will reduce aggregation and subsequent variability. I will also see if 70S fractions can be recovered from sucrose gradients and remain as 70S ribosomes. If this is the case, we may be able to run samples on gradients after lysis and purify ribosomes directly from the 70S fractions. I will test these methods of reducing ribosome aggregation and then use these ribosomes in *in vitro* assays to identify a preparation method that leads to the most reproducible activity from different ribosome samples. Reproducible activity will be assessed by comparing nLuc luminescence signal from technical replicates of purified ribosomes, signals from biological replicates of ribosomes purified on the same day, as well as ribosomes purified on different days. The homogeneity of ribosome preparations will be tested by evaluating peaks on sucrose gradient sedimentation

profiles. Finally, success will be determined by achieving a low degree of variability between preparations as well as low replicate to replicate variability in the *in vitro* assay.

D. METHODOLOGY AND PROCEDURES

Bacterial strains and growth conditions

The strain of *Francisella tularensis* used in these experiments is *F. tularensis* subsp. *holarctica* live vaccine strain (LVS), which is not infectious to humans, but retains its virulence in animal models. Bacteria are cultured in brain-heart infusion (BHI) medium supplemented with L-cysteine hydrochloride, β-NAD, heme-histidine, and 20% glucose (sBHIc) or on plates with cysteine-heart agar supplemented with 1% hemoglobin at 37°C. The MRE600 strain of *E. coli* used for ribosome purifications is cultured in LB liquid media or on plates with LB agar at 37°C. Strains with incorporated plasmids are selected for on kanamycin medium at 5 μg/mL for LVS or at 50 μg/mL for *E. coli*.

Plasmid design and construction

PCR primers were designed using SnapGene (GSL Biotech LLC) and ordered from ThermoFisher. Primers were used to amplify DNA fragments to be cloned into the intended plasmid, and the resulting DNA fragments were purified by washing and eluting through a spin column (QIAquick Purification Kit). The plasmid backbone and PCR fragments were digested using restriction endonucleases and subjected to gel electrophoresis. Desired products were excised and purified using the QIAquick Gel Extraction Kit. Ligations were conducted with T4 DNA ligase and the products were transformed into competent *E. coli* cells. Resulting colonies were used to

grow cultures, and the plasmid DNA was isolated using a QIAprep Spin Miniprep Kit and sequenced via Sanger sequencing at URI's INBRE CORE Facility.

70S ribosome purification

For *E. coli,* MRE600 cells were grown in 500 mL LB medium to mid-log phase (OD600 = 0.6-0.8), and for *F. tularensis*, wild-type LVS cells were grown in 500 mL sBHIc media to mid-log phase (OD600 = 0.5). Cells were chilled on ice for 20 min, centrifuged at 15,316 xg for 5 min at 4°C, then washed once with buffer $H^{10}M^{10}A^{50}$ (10 mM HEPES KOH pH 7.6, 10 mM $MgCl_2$, and 50 mM NH_4Cl), and centrifuged at 14,635 xg for 15 min at 4°C, and the resulting pellets were stored at -80°C. For ribosome purification, cell pellets were resuspended in 15 mL of $H^{10}M^{10}A^{50}$ with 20 U DNase I. Cells were either lysed by passing through a French press once at 800 lb/in^2 or by using Bug Buster detergent and incubating at 37°C for 1 h. Cell debris were removed by centrifugation at 146,000 xg for 15 min at 4°C. Supernatant was layered on top of $H^{10}M^{10}A^{500}$ + 20% sucrose (10 mM HEPES KOH pH 7.6, 10 mM $MgCl_2$, 500 mM NH_4Cl, 20% sucrose) and ribosomes were pelleted by ultracentrifugation in a 50.2 Ti rotor for 4 h at 146,000 xg at 4°C. The pellet was washed twice with $H^{10}M^{10}A^{50}$ and gently resuspended in $H^{10}M^{10}A^{50}$. This suspension was then layered onto another sucrose cushion ($H^{10}M^{10}A^{50}$ with 40% sucrose) and centrifuged for 14 h at 146,000 xg at 4°C to further purify the ribosomes. Purified ribosomes were gently resuspended in ~100 µL of $H^{10}M^{10}A^{50}$ and stored at -80°C.

Sucrose gradient sedimentation

A light solution of $H^{10}M^{10}A^{50}$ buffer + 10% sucrose was layered on top of a heavy solution of $H^{10}M^{10}A^{50}$ buffer + 50% sucrose in polycarbonate gradient tubes, and the gradients were mixed

in a BioComp Instruments 153 Gradient Station (BioComp). After refrigeration for 45 min the gradients were balanced, and 200 µL of sample was layered on top. The tubes were centrifuged in a Beckman-Coulter SW40 Ti rotor for 40,000 rpm for 4 h at 4°C and gradient fractions were collected using a Triax flow cell set to 260 nm. Thirty fractions were collected per run and stored at -80°C.

***In vitro* assays**

PureExpress Δ Ribosome Kit Protocol

Solution A and Factor Mix from the PureExpress Δ Ribosome Kit were thawed on ice. Ribosomes from sucrose cushion purification were diluted to 2.67 pmol/µL in $H^{10}M^{10}A^{50}$ buffer. Plasmid DNA was purified by phenol-chloroform extraction, and then diluted in 0.1x Buffer EB (Qiagen) to a concentration of 125 ng/µL. Each reaction contained 10 µL Solution A, 3 µL Factor Mix, and 2 µL template DNA and 15 µL ribosomes. After mixing gently and spinning briefly in a microfuge, the reactions were incubated at 37°C for 2 h in a thermocycler. The reactions were stopped by placing the tubes on ice, and the samples were used for analysis or frozen at –20°C for later use.

Nano-Glo® Luciferase Assay

Nano-Glo® Luciferase Assay Buffer and Nano-Glo® Luciferase Assay Substrate were thawed on ice. An appropriate volume of reconstituted reagent was prepared by combining one volume of substrate with 50 volumes of buffer. *In vitro* assay reactions were thawed on ice. Then, 30 µL of reconstituted reagent was added to the reactions, and the reactions were pipetted into a white 96-well plate. After a 3 min incubation period, the plate was read on the appropriate settings.

RESOURCES REQUIRED

Resources required for the completion of this project will come from the laboratory of Dr. Kathryn Ramsey. Sequencing services will be provided by the URI Genomic Sequencing Center.

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