EVALUATING *FRANCISELLA TULARENSIS* TRANSLATION *IN VITRO*

BY

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ABSTRACT

Francisella tularensis is a Gram-negative, facultative, intracellular bacterium which causes the disease tularemia. Previous research demonstrated that *F. tularensis* ribosomes are heterogeneous and that a specific ribosomal protein, bS21-2, regulates translation by interacting with the leader sequences of mRNAs (Trautmann and Ramsey 2022). Studies using reporter assays in bacterial cells (*in vivo*) made significant progress towards identifying leader sequences that confer regulation by bS21 (Trautmann et al. 2023), but we would like to recapitulate these results using purified ribosomes (*in vitro*) and develop a more comprehensive model of regulation by bS21.

The goal of my research was to develop an *in vitro* assay for translation using *F. tularensis* Live Vaccine Strain (LVS) ribosomes with the NEB PURExpress® *in vitro* translation kit. To achieve this goal, I developed a sensitive and easily modifiable reporter construct, purified active ribosomes from *Escherichia coli* and *F. tularensis* LVS, and optimized the reproducibility of the *in vitro* assay.

For my research project, I used standard cloning techniques to generate a DNA template for the *in vitro* assay that encodes a luminescent reporter and is sensitive and easy to modify. I also purified salt-washed *E. coli* ribosomes and determined that they were translationally active in the assay. Then, I replicated these results using ribosomes from multiple purification attempts, validating that we can reproducibly isolate active ribosomes. In addition, I demonstrated that *E. coli* ribosomes are inhibited by ribosome-targeting antibiotics, indicating

that we can use the assay to measure translation inhibition. Next, I purified ribosomes from *F. tularensis* and made enhancements to increase ribosome concentration and yield. Then, I found that *F. tularensis* ribosomes appeared to aggregate more readily than *E. coli* ribosomes and that this aggregation seemed to impact translational activity. After further testing, I modified the amount of *F. tularensis* LVS ribosomes used in the *in vitro* assay to reduce aggregation and obtain a significant and reproducible signal.

The optimized *in vitro* assay that I developed will be used in future research to measure translation efficiency of mRNA leader sequences by *F. tularensis* ribosomes with specific homologs of bS21 and will contribute to a better understanding of how bS21 homologs regulate translation in *F. tularensis.*

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CHAPTER 1

INTRODUCTION & REVIEW OF LITERATURE

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, Sjostedt, and Titball 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 a type VI secretion system (T6SS). In *F. tularensis*, the T6SS is necessary for bacterial intracellular survival and growth within host cells. Expression of the FPI 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 (Cuthbert et al. 2017; Ramsey et al. 2015; Travis et al. 2021). 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 and 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 of 16S rRNA 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 and Wijnands 1981; Chang and 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 and Ramsey 2022). Additionally, the loss of bS21-2 leads to decreased abundance of certain proteins, including T6SS proteins, yet mRNA abundance remains the same (Trautmann and Ramsey 2022). This suggests that when bS21-2 is absent, ribosomes cannot translate these mRNAs as efficiently. We also found that the ability of cells to replicate within macrophage decreased when bS21-2

was absent (Trautmann and 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, specifically the promoter for *F. tularensis* gene *tul4* (*tul4* is not regulated by bS21-2 (Trautmann and Ramsey 2022)), driving the transcription of an mRNA containing the 5′ UTR of a control gene or gene of interest fused to a superfolder green fluorescent protein (sfGFP) coding sequence. sfGFP fluorescence 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 lower protein abundance corresponding to the selected genes (Trautmann and Ramsey 2022). These genes included the FPI genes *pdpA* and *iglA*. When bS21-2 was absent, reporter output decreased when fused to the 5′ UTRs of the selected genes but not when fused to the 5′ UTR of *tul4* (**Figure 1**; Trautmann and 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.

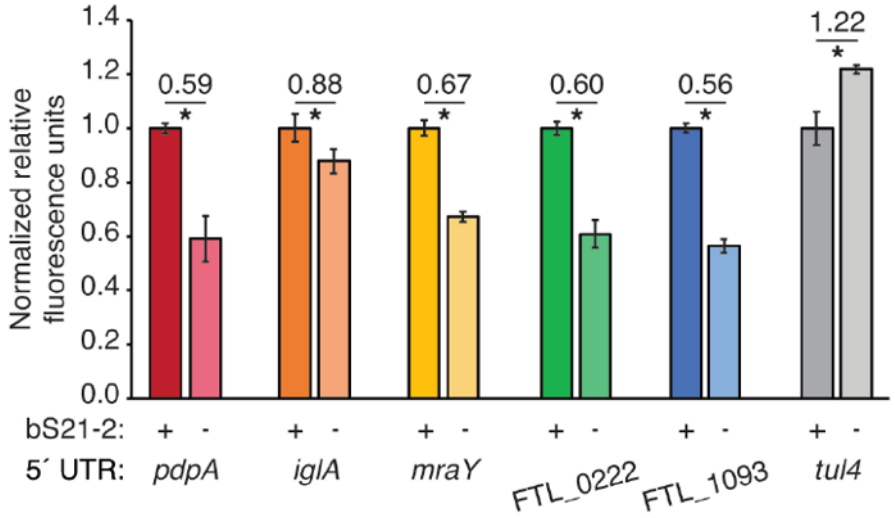

Figure 1. 5′ UTRs are sufficient to lead to bS21-2-mediated changes in translation. Relative fluorescence for indicated GFP translational fusion reporters in cells with (+; WT) or without (−; Δ*rpsU2*) bS21-2. The *tul4* 5′ UTR serves as a control. Lines above bars indicate comparisons, values above 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. 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 which elements of 5′ UTR sequences 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 continued.

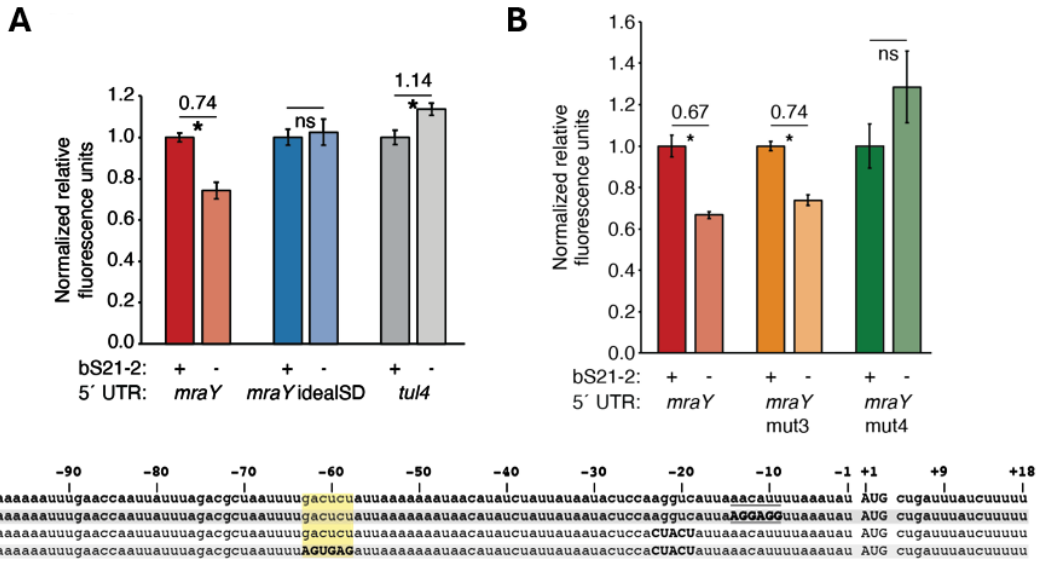

Figure 2. **bS21-2-mediated translation of *mraY* depends on a weak Shine-Dalgarno and a specific six nucleotide sequence.** Charts show relative fluorescence for indicated *gfp* translation fusion reporters in cells with (+; WT) or without (−; Δ*rpsU2*) bS21-2. (A) Introduction of an ideal SD in the *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 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 was to develop an *in vitro* translation assay for *F. tularensis*. In developing this assay, I used 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 cloned a plasmid template and purified active ribosomes from *F. tularensis*.

Preliminary work in developing an *in vitro* translation assay, performed by Hannah Trautmann, used *F. tularensis* ribosomes and the *tul4* 5′ UTR – sfGFP reporter. While the reaction with manufacturer-provided *E. coli* ribosomes yielded a signal in the assay, demonstrating that the reaction was properly set up, the ribosomes purified from *F. tularensis* did not translate detectable amounts of product (**Figure 3**). These results suggested that we try to find a more sensitive reporter and ensure that the purified ribosomes are sufficiently active.

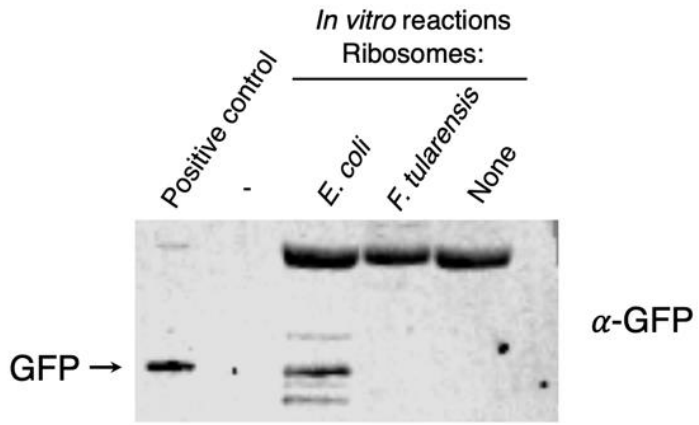

Figure 3. Ribosomes purified from *F. tularensis* exhibit poor activity in initial *in vitro* assays, as shown in Western blot. *In vitro* translation assays were performed using a DNA template specifying an mRNA with the *tul4* 5′ UTR fused to the sfGFP gene. Reactions contained either control *E. coli* ribosomes provided in the kit, 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 sfGFP. The positive control lane contains cell lysate from *F. tularensis* expressing sfGFP. Intense bands near the top of the gel indicate non-specific binding.

My thesis project is part of a larger effort to understand how bS21 controls translation of mRNAs with specific 5′ UTRs. The purpose of this thesis was to develop an *in vitro* assay for *F. tularensis,* which could assess the translation efficiency of various genes, test for sequence motifs that confer regulation by bS21, and assist in developing a more comprehensive model of regulation by bS21. For my thesis, I cloned a plasmid template to use in the *in vitro* assay that was sensitive and easy to modify. I also purified reproducibly active ribosomes from *E. coli* and *F. tularensis*. I then used the plasmid template and purified ribosomes with the PURExpress® Δ Ribosome Kit to measure translation efficiency of specific 5′ UTRs. I found that *in vitro* translation efficiency was dependent on bacterial species, method of lysis, and the amount of ribosomes loaded in the assay. In addition, I validated and demonstrated that *E. coli* ribosomes are sensitive to kasugamycin, an aminoglycoside that inhibits translation and binds near bS21, demonstrating that the *in vitro* assay can be used to test for translation inhibition by specific antibiotics.

CHAPTER 2

METHODOLOGY

Bacterial strains and growth conditions

Francisella tularensis subsp. *holarctica* LVS cells were cultured at 37°C in supplemented Mueller-Hinton broth or in supplemented brain heart infusion broth with cysteine, shaking aerobically, or on plates with cysteine heart agar supplemented with 1% hemoglobin (CHAH). Mueller-Hinton broth was supplemented with 1 mg/mL glucose, 0.25 mg/mL iron pyrophosphate, and 2% Isovitalex (sMHB). Brain heart infusion broth was supplemented with 1 mg/mL cysteine, 0.01 mg/mL β-NAD, 0.01 mg/mL heme-histidine, and 5 mg/mL glucose (sBHIc). *Escherichia coli* strain MRE600 (Kurylo et al. 2016) was used for ribosome purifications, and *E. coli* strain XL1 Blue was used for plasmid cloning and propagation. *E. coli* cells were grown at 37°C, shaking aerobically in LB liquid media or on LB agar. *E. coli* cells harboring replicating plasmids were selected for on carbenicillin medium at 100 μg/mL.

Plasmid construction

The plasmids constructed for this study were derived from the DHFR control plasmid provided in the NEB PURExpress® transcription/translation system kit. The DHFR plasmid, which has a high copy number pUC origin and encodes ampicillin resistance, also encodes the bacteriophage T7 RNA polymerase promoter driving expression of the *E. coli* dihydrofolate reductase (DHFR) gene. The plasmid also contains a ribosome binding site (RBS) which increases ribosome binding efficiency during translation and a multiple cloning site (MCS) downstream of the DHFR gene for easy modification. With these

elements in place, the DHFR gene can easily be exchanged with a gene of interest and transcribed and/or translated in the *in vitro* system.

The intermediate plasmid pKR81 is derived from the control DHFR plasmid, modified to include DNA specifying the T7 promoter driving expression of an *F. tularensis* control gene, *tul4*. The modified DNA was generated by synthesis (IDT). This fragment encodes the T7 promoter directly followed by 536 bp of DNA from *F. tularensis*, which includes the *tul4* transcription start site (36 bp upstream of the translation start site; Zaide et al. 2011) and extends 50 bp past the *tul4* stop codon and encodes BmtI digestion sites on both ends. pKR81 was constructed by digesting the control DHFR plasmid with BmtI to remove the T7 promoter and DHFR gene and cloning in the digested synthesized DNA fragment.

The pKR144 plasmid was derived from pKR81 with the addition of a second, divergently positioned T7 promoter driving expression of the 5′ untranslated region (5′ UTR) of the *F. tularensis* gene *pdpA* followed by the coding sequence for the small luciferase gene NanoLuc (nLuc; Hall et al. 2012). The *pdpA* 5′ UTR includes the transcription start site (24 bp upstream of the translation start site) and the first six codons (Ramsey et al. 2015). This additional DNA was generated as a fragment by synthesis (IDT), which also included DNA specifying a SalI site on the 5′ end and a PvuII site on the 3′ end. pKR144 was cloned by digesting pKR81 with SalI and PvuII, which removed 11 bp, and ligating in the digested synthesized DNA fragment.

The pKR204 and pKR205 plasmids were ultimately derived from pKR144. To generate pKR204, pKR144 was modified such that, between the *pdpA* 5′ UTR and nLuc, DNA specifying a NotI site and one extra bp was added, encoding an in-frame alanine linker. The sequence following the other T7 promoter was also modified. After the *tul4* 5′ UTR and the first six *tul4* codons, DNA was added that specifies a NotI site and one extra bp, encoding an in-frame alanine linker, followed by the coding sequence for the fluorescent protein LanYFP. The pKR205 plasmid only differs from pKR204 in that, after the NotI site following the *tul4* 5′ UTR, it encodes the fluorescent protein iLov.

The pKR214 plasmid, ultimately derived from pKR204, only encodes a single T7 promoter driving expression of the *tul4* 5′ UTR followed by nLuc. The *tul4* 5′ UTR includes the *tul4* transcription start site and the first six codons of *tul4* and is followed by DNA specifying a NotI site and one extra bp, encoding an in-frame alanine linker. The *tul4* 5′ UTR – nLuc fusion was generated by ligating together PCR products generated with NotI sites encoded in primers. The second T7 promoter and reporter encoded on pKR144 was removed by digestion with SalI and XhoI and ligation of the compatible ends.

Plasmids were validated by Sanger sequencing at URI's INBRE Molecular Informatics Core.

Table 1. Plasmids used in this study.

Plasmid	Reporter	Additional Notes / Features
PURExpress® Control DHFR Plasmid	T7–*E. coli* dihydrofolate reductase (DHFR)	Control template from NEB PURExpress® kit, encodes ampicillin resistance, pUC origin of replication

pKR81	T7–UTR$_{tul4}$–*tul4*	Derived from PURExpress® Control DHFR Plasmid
pKR144	T7–UTR$_{pdpA}$–nLuc, T7–UTR$_{tul4}$–*tul4*	Derived from pKR81
pKR204	T7–UTR$_{pdpA}$–nLuc, T7–UTR$_{tul4}$–LanYFP	Includes alanine linker / NotI cut site between *pdpA* 5′ UTR and nLuc Derived from pKR144
pKR205	T7–UTR$_{pdpA}$–nLuc, T7–UTR$_{tul4}$–iLov	Includes alanine linker / NotI cut site between *pdpA* 5′ UTR and nLuc Derived from pKR144
pKR214	T7–UTR$_{pdpA}$–nLuc	Includes alanine linker / NotI cut site between *pdpA* 5′ UTR and nLuc Derived from pKR204

70S ribosome purification

Half a liter of either *E. coli* MRE600 or *F. tularensis* LVS cells were grown to mid-log phase (OD_{600} 0.6-0.8 or 0.5, respectively). Cells were chilled on ice for 20 min, pelleted by centrifugation at 15,316 × *g* for 5 min at 4°C, washed with buffer $H^{10}M^{10}A^{50}$ (10 mM HEPES KOH pH 7.6, 10 mM $MgCl_2$, and 50 mM NH_4Cl), pelleted by centrifugation at 14,635 × *g* for 15 min at 4°C, and stored at –80°C. Cells were resuspended in 15 mL of $H^{10}M^{10}A^{50}$ with 20 U DNase I and either lysed by passing through a French press once at 800 lb/in^2 or by including BugBuster® Protein Extraction Reagent (MilliporeSigma) in the resuspension buffer at 1x concentration and incubating at 37°C for 1 h. Cell debris were removed by centrifugation at 81,800 × *g* 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 × *g* 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 81,800 × *g* at 4°C. Purified ribosomes were gently resuspended in 100 µL of either $H^{10}M^{10}A^{50}$ or low magnesium buffer ($H^{10}M^{0.3}A^{50}$) at 4°C overnight. Several initial A260 readings were taken, averaged, and used to calculate ribosome concentration. For samples in low magnesium buffer, sufficient 1 M $MgCl_2$ was added to bring the magnesium to a concentration of 10 mM, and the samples were incubated on ice for at least two hours. Finally, ribosome samples were diluted in $H^{10}M^{10}A^{50}$ buffer to 2.67 pmol/µL, aliquoted in 16 µL volumes, 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 open polyclear gradient tubes (Seton), and the gradients were made using a Gradient Station (BioComp). Tubes with gradients were refrigerated for 45 min, 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 hours at 4°C. The nucleic acid content throughout the gradients were assessed using a Triax flow cell set to 260 nm. Thirty fractions were collected per gradient using a Gilson Fraction Collector (FC-203B) and stored at –80°C.

***In vitro* translation**

Ribosomes, Solution A, and Factor Mix from the PURExpress® Δ Ribosome Kit (NEB) were thawed on ice. Templates were phenol-chloroform-

purified plasmid DNA. Reactions were assembled using a master mix; individual reactions contained 10 µL Solution A, 3 µL Factor Mix, 250 ng template, and 40 pmol ribosomes in a total volume of 30 µL. To measure inhibition of translation by antibiotics, reactions were prepared in a total volume of 29 µL, and 1 µL of diluted antibiotic was added to reach final concentrations of 0.005 mM, 0.05 mM, or 1 mM. Thiostrepton was diluted in DMSO to 50 mg/mL, and kasugamycin was diluted in water to 50 mg/mL and then used to prepare 30 mM, 1.5 mM, and 0.15 mM stocks. Reactions were mixed gently and incubated at 37°C for 2 hours. The reactions were stopped by placing the tubes on ice, and samples were used for a Nano-Glo® Luciferase Assay or frozen at –20°C for later use.

Nano-Glo® Luciferase Assay

In vitro translation reactions, Nano-Glo® Luciferase Assay Buffer, and Nano-Glo® Luciferase Assay Substrate (Promega) were thawed on ice. Assay substrate was diluted 1:50 with Assay Buffer and mixed with an equal volume of individual translation reactions. Reactions were incubated at room temperature for 3 minutes, and luminescence was measured in a white 96-well plate.

Growth curves

F. tularensis LVS cells were grown on CHAH plates at 37°C overnight. Cells were resuspended in sMHB media and used to inoculate triplicate cultures of either sMHB media or sBHIc media (OD_{600} 0.08). Cultures were

grown aerobically at 37°C, and growth was measured by assessing cell density (OD600) at specific time intervals.

RNA extraction and analysis

RNA was extracted either following the RNA-Snap protocol (Stead et al. 2012) or using the Direct-zol RNA Miniprep kit (Zymo Research) according to the manufacturer's protocol. RNA samples were stored at –80°C.

RNA samples were analyzed by agarose gel electrophoresis. A total of 2.5 µg each of the Ambion® Millennium™ Markers-Formamide RNA ladder and RNA samples were treated with NorthernMax™-Gly Sample Loading Dye according to the manufacturer's protocol and separated on a 1% agarose gel prepared with NorthernMax™ Gly Gel Prep/Running Buffer.

CHAPTER 3

FINDINGS

Evaluating translation *in vitro* using ribosomes from *F. tularensis*

The primary goal of my research project was to develop an *in vitro* assay for translation using *F. tularensis* Live Vaccine Strain (LVS) ribosomes. To achieve this goal, I worked with the commercially available NEB PURExpress® Δ Ribosome *in vitro* translation kit. This kit contains all the factors necessary for transcription and translation with user-provided ribosomes.

Reporter development for *in vitro* translation

My first task was to develop a reporter template for *in vitro* transcription and translation that produced a high level of signal and was easy to modify. A former lab member had tested a template encoding the commonly used fluorescent reporter, superfolder green fluorescent protein (sfGFP), but it did not yield a detectable signal when translated by *F. tularensis* LVS ribosomes (**Ch 1, Fig 3**). With this in mind, I considered other fluorescent and luminescent reporters, evaluating their size, brightness, and emission spectra.

I tested two fluorescent reporters, LanYFP and iLov, to determine if they exhibited a stronger fluorescent signal than sfGFP and might be detectable after translation by *F. tularensis* ribosomes. Specifically, I cloned the genes encoding each fluorescent reporter into a plasmid used as a template for *in vitro* translation (pKR144) to make a plasmid encoding LanYFP (pKR204) and another encoding iLov (pKR205). I compared the fluorescence produced by

each reporter plasmid when maintained in *E. coli* cells, and I included *F. tularensis* LVS cells with a plasmid encoding the sfGFP reporter (plasmid pKR146; Trautmann et al. 2023) for reference (**Figure 1A**). In this experiment, I found that LanYFP and iLov produced similar strong, measurable signals (**Figure 1A**). Since the filters we had in the spectrophotometer were more closely aligned with the excitation and emission wavelengths for LanYFP, I decided to continue testing LanYFP.

To assess the signal strength from LanYFP compared to a luminescent reporter, I generated a plasmid template which encoded both LanYFP and the small luciferase nLuc (pKR204). I used this template in an *in vitro* translation reaction with ribosomes purified from either *E. coli* or *F. tularensis* LVS and compared the signal strength (**Figure 1B**). I found that the signal from nLuc was almost an order of magnitude higher than the signal for LanYFP when translated by LVS ribosomes and even higher when translated by *E. coli* ribosomes. Thus, nLuc was selected for the remaining experiments because of its high signal strength compared to the other reporters.

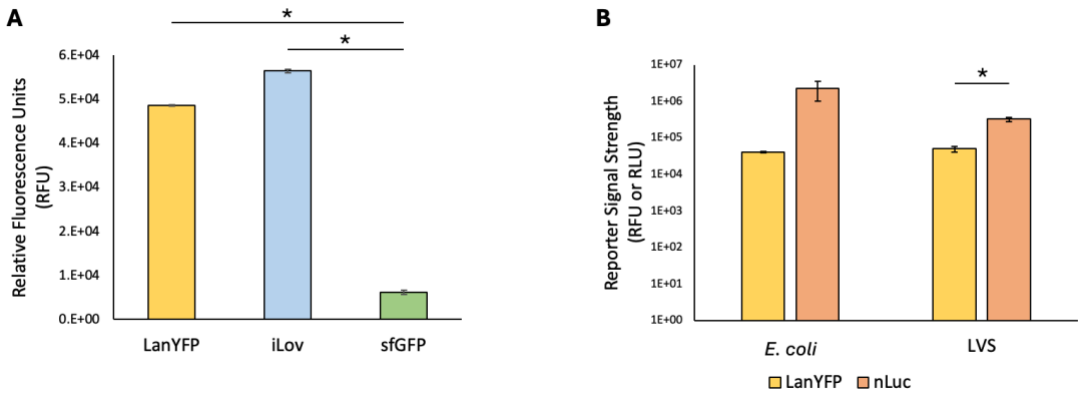

Figure 1. Comparison of reporters demonstrates that nLuc has the highest signal strength of reporters tested. (A) Comparison of signal from

fluorescent reporters detected in cells. Chart shows relative fluorescence for indicated translation fusion reporters LanYFP (pKR204), iLov (pKR205), and sfGFP (pKR146). This experiment was performed in technical triplicate. (B) Comparison of LanYFP fluorescence and nLuc luminescence. Reporter signal strength is displayed in relative fluorescence units (RFU) or relative luminescence units (RLU) for the translation fusion reporter (pKR204) 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. This experiment was performed in biological duplicate. Error bars represent 1 SD. Lines above bars indicate comparisons. *$P < 0.05$ by *t*-test.

Having identified a reporter with high signal strength, I cloned a plasmid template for *in vitro* translation with a single reporter that would allow the 5′ untranslated region to be easily altered. Specifically, I modified the plasmid with both nLuc and LanYFP (pKR204) so that it only encodes the 5′ untranslated region (UTR) of *tul4*, a control gene with strong translation in LVS, followed by nLuc (pKR214, **Figure 2**). The 5′ UTR of *tul4* can be easily removed and then exchanged with another 5′ UTR after digestion with NotI and XmaI restriction enzymes. The reporter is sensitive, because nLuc consistently displayed the highest signal ($3.0x10^5$ – $3.2x10^6$ RLU) of all the reporters we tested, and it is easy to modify, because the two enzyme digestion sites (NotI and XmaI) allow for easy replacement of the UTR–promoter region with a DNA fragment encoding a different UTR.

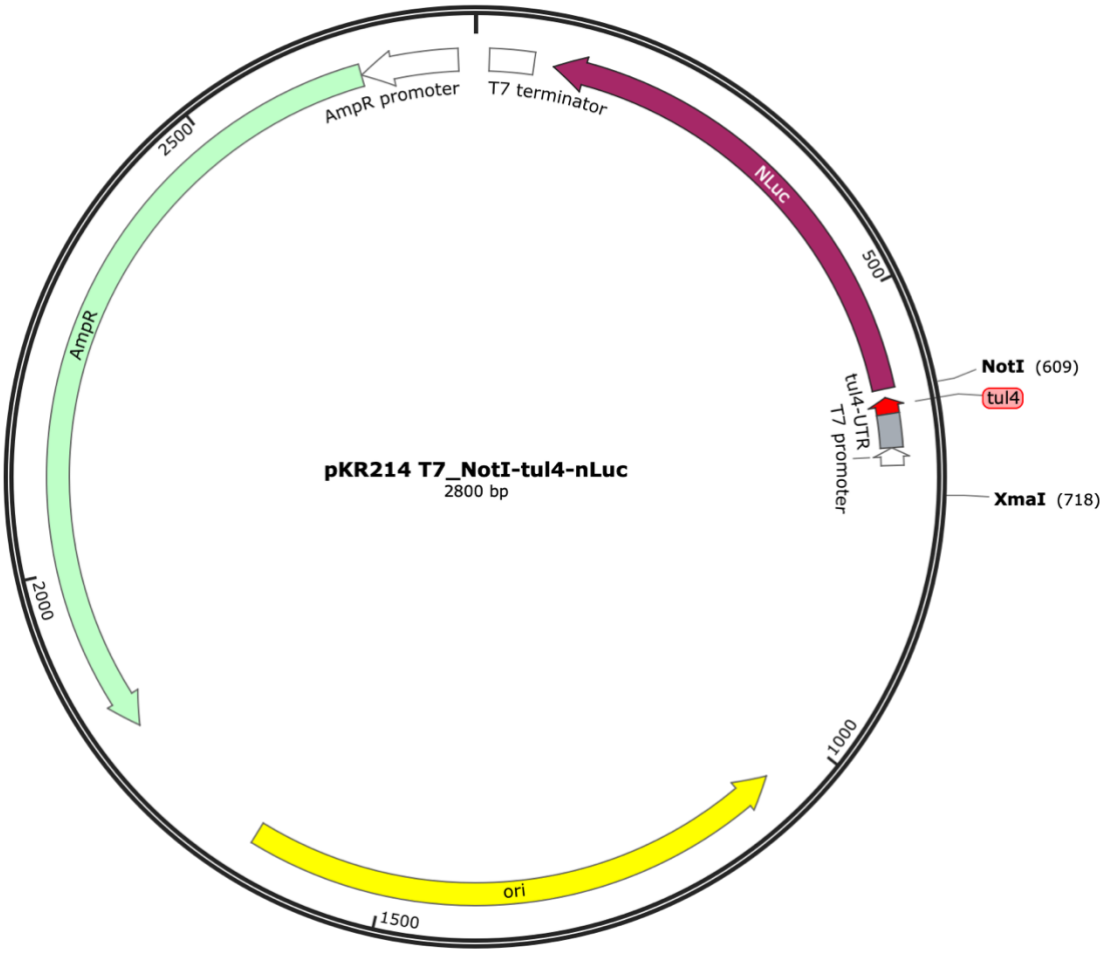

Figure 2. A sensitive and easily modifiable reporter plasmid for testing translation of 5′ UTRs. Diagram of the nLuc reporter plasmid, pKR214 PT7-*tul4* 5′ UTR-*nLuc*, encoding *nLuc* with the 5′ UTR of *tul4*. The T7 promoter and 5′ UTR are flanked by NotI and XmaI restriction enzyme cut sites, allowing for easy modification.

Purification of active ribosomes from *E. coli*

The second goal of my research was to purify active salt-washed ribosomes from *E. coli* and *F. tularensis* LVS using a sucrose cushion purification method. I began by validating this method with *E. coli* MRE600, a strain which lacks RNase I and is commonly used for ribosome purifications (Kurylo et al. 2016). In this project, *E. coli* served as a positive control for ribosome isolation and activity. I harvested ribosomes from *E. coli* cells grown

in LB medium and was able to achieve a yield of 1.5-5 nmol of ribosomes from 1 L of culture (at a concentration of 10-30 pmol/µL). This was substantially higher than the minimum of 40 pmol ribosomes (at a concentration of at least 2.7 pmol/µL) used in 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 (**Figure 3**). The *E. coli* MRE600 ribosomes I purified, when used in *in vitro* assays, had similar activity to kit-provided *E. coli* ribosomes and resulted in reasonably similar reporter signal (within a log-fold difference) across purifications. This demonstrated that our sucrose cushion purification protocol yields reproducibly active *E. coli* ribosomes.

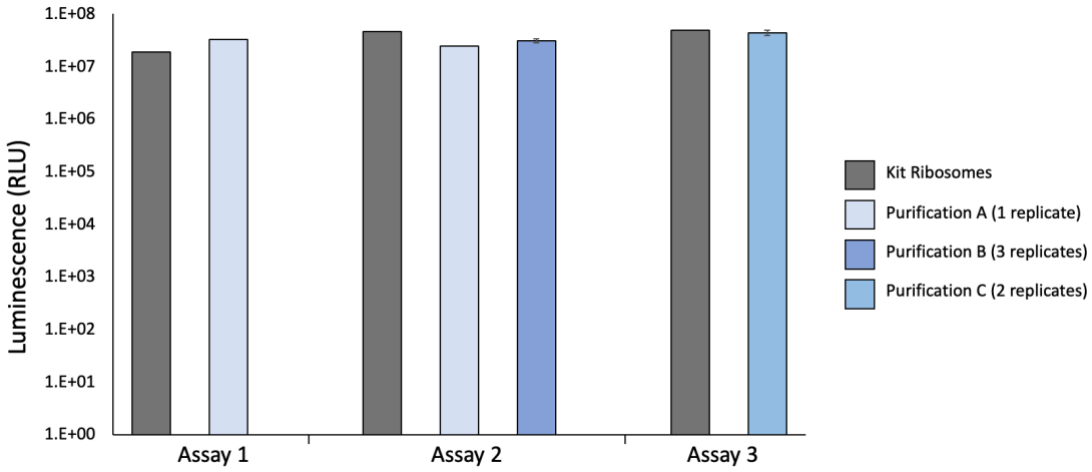

Figure 3. Relative luminescence produced by nLuc translated by purified *E. coli* ribosomes shows reproducible ribosome activity. Results of *in vitro* translation assays using the *pdpA* 5′ UTR-*nLuc* reporter (pKR144) showing translation efficiency of multiple *E. coli* (Ec) ribosome purifications tested on different days. Salt-washed ribosomes were purified using the sucrose cushion method. Kit ribosomes are also from *E. coli* and were supplied with the NEB assay kit.

Activity of ribosome-targeting antibiotics can be measured *in vitro*

Having validated that we can purify active *E. coli* ribosomes, we wanted to see if the *in vitro* translation assay could be used to assess inhibition of translation by ribosome-targeting antibiotics. We chose to test two antibiotics, thiostrepton, a strong antibiotic previously studied *in vitro* (Asikaer, Sun, and Shen 2024), and kasugamycin, a weaker antibiotic. Thiostrepton is a thiopeptide antibiotic that binds to the large subunit of the ribosome and inhibits translation by interfering with the L11 ribosomal protein (Bailly 2022). Kasugamycin is an aminoglycoside of interest to us because it binds near a protein studied in the Ramsey lab, bS21, on the ribosome. To assess inhibition, I added the indicated antibiotic to the *in vitro* translation reaction and measured reporter activity (**Figure 4**). I found that, in this experiment, kasugamycin inhibited translation of *E. coli* ribosomes at a concentration of 1 mM, and thiostrepton inhibited translation at a concentration of 1 mM even more significantly than kasugamycin. Thus, we concluded that we could measure translation inhibition by antibiotics in our *in vitro* assay.

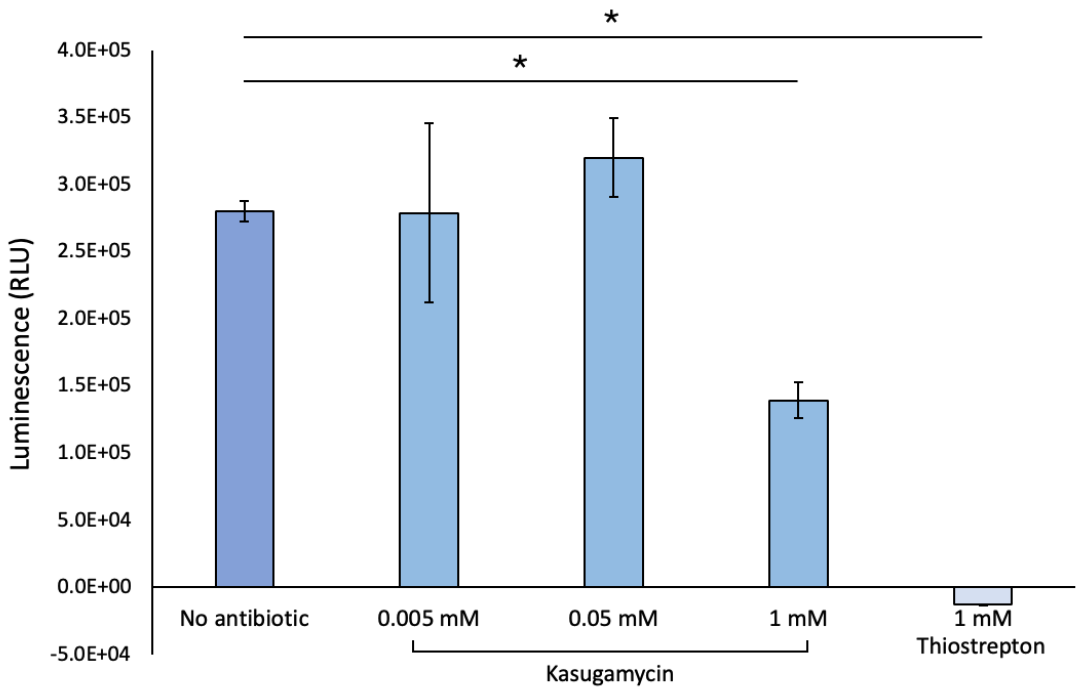

Figure 4. *In vitro* translation can be inhibited by ribosome-targeting drugs. A concentration of 1 mM kasugamycin or thiostrepton limits nLuc activity, indicating translation inhibition. Reporter signal strength is displayed in relative luminescence units (RLU). Samples with 1 mM thiostrepton were negative because signal was below that of background. This experiment was performed in technical triplicate. This experiment was performed twice, and the results are representative. Error bars represent 1 SD. Lines above bars indicate comparisons. *$P < 0.05$ by *t*-test.

Purification of active ribosomes from *F. tularensis*

After validating that salt-washed ribosomes isolated using the sucrose cushion method yielded active *E. coli* ribosomes, I purified ribosomes from *F. tularensis* LVS cells grown in supplemented Mueller-Hinton broth (sMHB). Initial purification attempts were successful but yield and concentration were low (~0.9 pmol/µL), so subsequent efforts focused on increasing yield. I doubled the volume of bacterial cells (to one liter for each sample), and I tested an alternate growth medium, supplemented brain heart infusion broth with cysteine (sBHIc). Specifically, I wanted to see if *F. tularensis* LVS would

reach mid-log phase at a higher OD600 in sBHIc than in sMHB, which would be advantageous because more cells should yield more ribosomes. *F. tularensis* LVS cultures were started in sMHB and sBHIc media at an OD600 of ~0.08 and grown in a shaking incubator at 37°C while optical density was monitored (**Figure 5**).

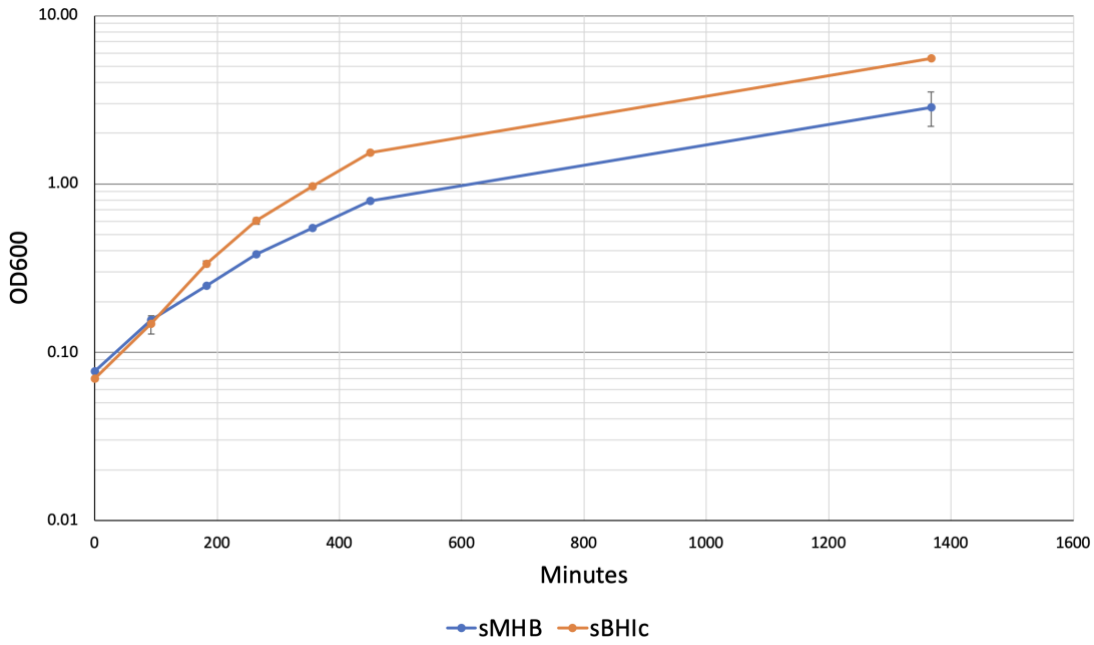

Figure 5. *F. tularensis* LVS grows faster and to a higher density in sBHIc medium than in sMHB medium. Each point represents the average of two technical replicates. This was also repeated with LVS harboring an empty vector and is representative of both experiments. Error bars represent 1 SD.

I found that *F. tularensis* LVS grew faster and maintained mid-log growth at higher cell densities in sBHIc medium than in sMHB medium. Because harvesting mid-log *F. tularensis* LVS cells at a higher density (OD600 of ~0.5 in sBHIc compared to ~0.3 in sMHB) recovers more cell mass, I proceeded to grow *F. tularensis* LVS in sBHIc medium for all further purifications. Doubling the volume of cultured cells and switching to sBHIc medium led to a sizable

increase in yield (~1.9 nmol ribosomes at ~16 pmol/µL), allowing me to obtain enough *F. tularensis* LVS ribosomes for *in vitro* assays.

Next, I wanted to assess the consistency and reproducibility of *E. coli* and *F. tularensis* LVS ribosome activity. I took several steps to reduce variability and enhance consistency across assays, namely resuspending ribosome pellets overnight to ensure homogeneity, averaging several measures of concentration to increase accuracy, storing single-use aliquots to reduce freeze/thaw cycles, and using an assay master mix to reduce pipetting error. I tested reproducibility by comparing nLuc luminescence signal from *in vitro* assays using different ribosome purifications and multiple technical replicates (**Figure 6**). While I observed reproducible translation with *E. coli* ribosomes, there was a significant degree of variability in translation by *F. tularensis* ribosomes, including variability between technical replicates.

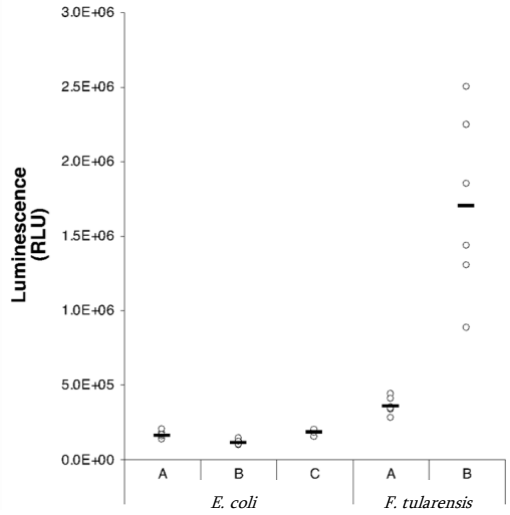

Figure 6. Assessment of translation by ribosomes from *E. coli* and *F. tularensis* shows variability in signal for *F. tularensis* ribosomes. Results of *in vitro* translation assays using the *tul4* 5´ UTR-nLuc reporter (pKR214) and indicated ribosomes. Letters indicate biological replicate purifications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

Given the difference in reproducibility between ribosomes isolated from *E. coli* and *F. tularensis* LVS, I also analyzed ribosome samples on sucrose gradients. My goal was to see if their gradient profiles would help us

understand the observed variability of *F. tularensis* LVS ribosome activity in *in vitro* assays. I found that purified *E. coli* ribosomes exhibit a prominent peak corresponding to 70S ribosomes but also have a small peak at lower density that likely represents 50S subunits, along with higher density peaks that likely correspond to polysomes (**Figure 7**). When analyzing *F. tularensis* ribosomes, I similarly found a distinct 70S ribosome peak. However, I also observed a lower-density shoulder on the 70S peak and a single higher-density peak that may represent aggregated 70S ribosomes or 100S ribosome dimers. Ribosomes form 100S dimers when two 70S ribosomes interact with hibernation factors and undergo conformational changes; this is usually accompanied by downregulation of translation (Prossliner et al. 2018). This analysis suggests that in our *F. tularensis* ribosome purifications, we have active 70S ribosomes along with either 70S aggregates or 100S ribosomes. Thus, in each sample there may be a subset of ribosomes that are inactive, potentially leading to the observed variability.

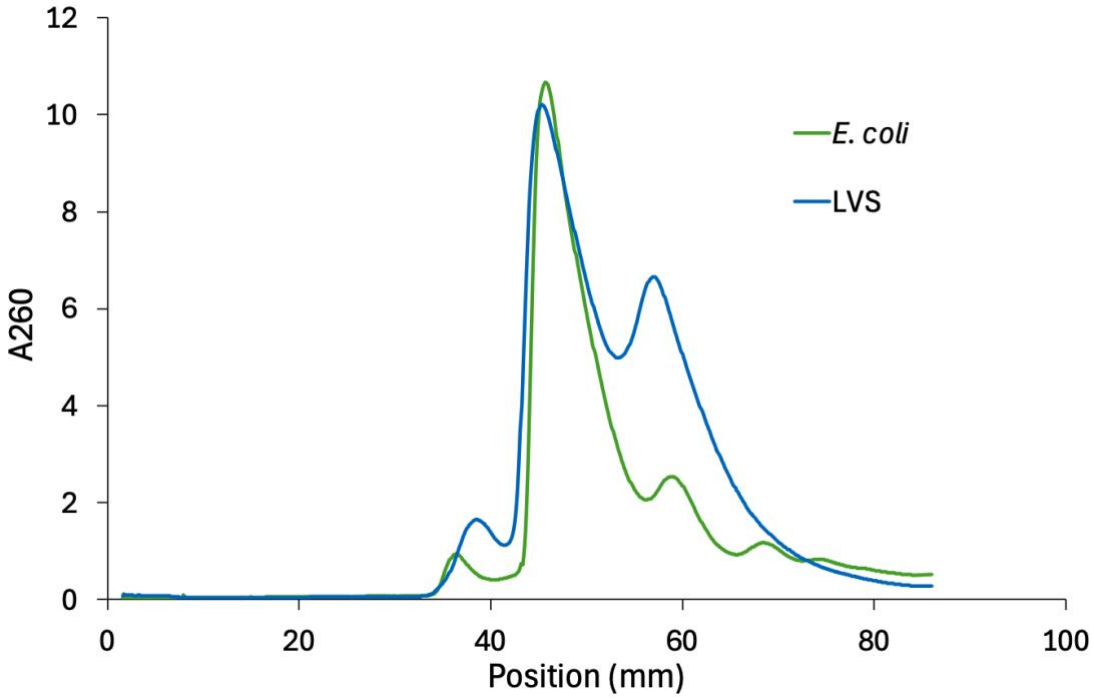

Figure 7. Sucrose gradient sedimentation profiles reveal differences in composition between *E. coli* and *F. tularensis* LVS ribosomes. Sucrose gradient profiles of ribosomes isolated from either *E. coli* (green) or *F. tularensis* LVS (blue). Salt-washed ribosomes were purified using the sucrose cushion method after cells were lysed with BugBuster®, and a total of 570-590 pmols were used in these gradients. Data from representative experiments are shown.

Lower *F. tularensis* ribosome concentration leads to sharper 70S peaks

Having analyzed multiple ribosome samples using sucrose gradients, I noticed that sucrose gradients with lower concentrations of *F. tularensis* LVS ribosomes tended to have sharper peaks corresponding to 70S particles. I chose to test how total amounts of ribosomes impacted the gradient profiles. Specifically, I analyzed sucrose gradients loaded with 200, 800, or 1400 pmol of *F. tularensis* LVS ribosomes (**Figure 8**). I found that loading fewer ribosomes leads to sharper 70S peaks and less of a higher-density shoulder.

This suggests to us that when there is a lower concentration of *F. tularensis* LVS ribosomes in a sample, there may be less aggregation or clumping.

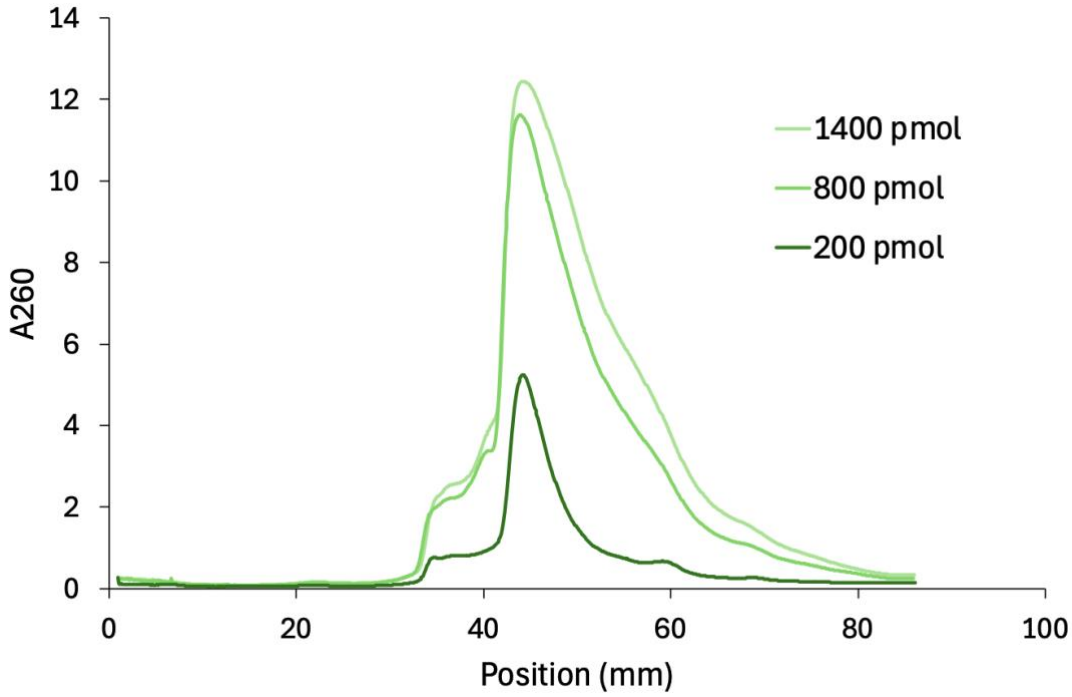

Figure 8. Loading fewer *F. tularensis* ribosomes onto sucrose gradients results in less aggregation. Profiles of sucrose gradients loaded with indicated amounts of *F. tularensis* LVS ribosomes from the same sample. Salt-washed ribosomes were purified using the sucrose cushion method after cells were lysed by French press.

Ribosome concentration affects translation efficiency

Since we observed that higher concentrations of *F. tularensis* ribosomes can lead to aggregation, I hypothesized that changing the concentration of ribosomes in the *in vitro* translation assay might impact translation activity. Thus, I performed an experiment to measure reporter signal (luminescence) per ribosome at different ribosome concentrations, with the highest amount being the kit-recommended minimum of 40 pmol. I also used several different ribosome samples, specifically the kit-provided *E. coli* ribosomes or *F.*

tularensis LVS ribosomes from cells lysed either by French press or the commercially available detergent BugBuster® (**Figure 9**). For all three ribosome samples, I found that lower amounts of ribosomes exhibited higher luminescence per pmol of ribosomes, with the highest per-ribosome signal from reactions containing 2.5 pmol or fewer ribosomes. This effect is most dramatic for the *E. coli* ribosomes, and the signal per ribosome at lower concentrations is significantly higher for *F. tularensis* LVS ribosomes purified from cells lysed by French press than by BugBuster®. Together, these experiments suggest that higher concentrations of ribosomes may lead to aggregation and variability, while lower ribosome concentrations may produce consistent and reproducible signals *in vitro*.

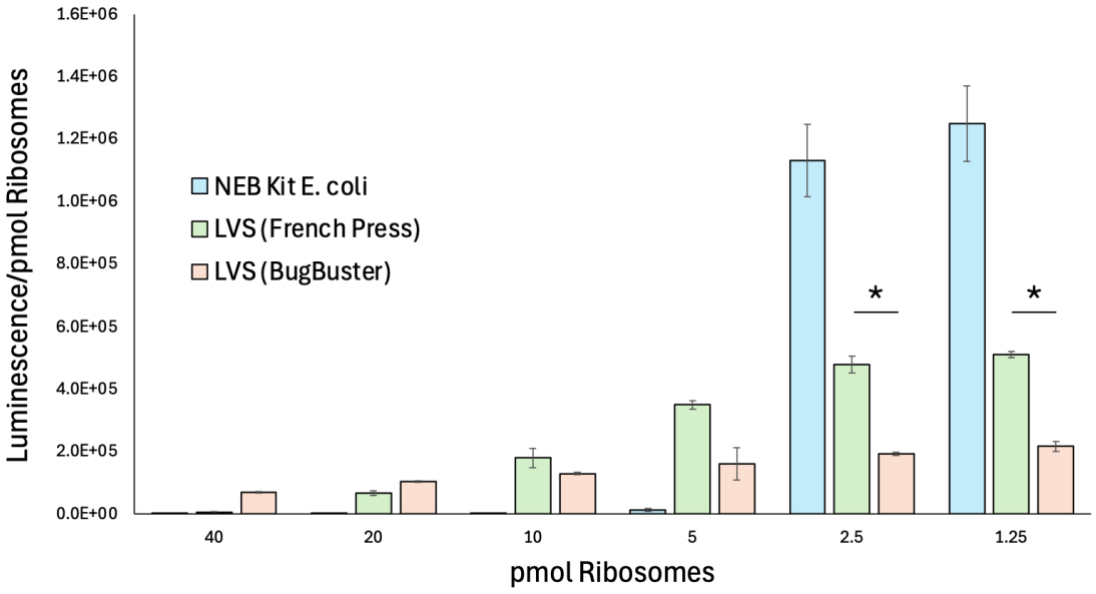

Figure 9. Lower ribosome concentrations in the *in vitro* translation assay leads to higher signal per ribosome. Chart depicts relative efficiency of *in vitro* translation assay using pKR214 as a template and indicated amount and type of ribosomes. Experiment was performed in technical triplicate. Error bars represent 1 SD. Lines above bars indicate statistical comparisons. *$P < 0.05$ by *t*-test.

F. tularensis ribosomes isolated from cells lysed by French press, not BugBuster®, translate more reporter protein

When assessing how *F. tularensis* ribosome concentration impacts translation, I found that using about 2 pmol of LVS ribosomes yields the highest signal-to-ribosome ratio. But, I also found that the method by which cells are lysed prior to ribosome purification appears to affect translation efficiency. This led me to test if using 2 pmol ribosomes in each reaction would give a measurable and consistent signal and if there would be a consistent difference between ribosomes purified from cells lysed by French press or with BugBuster®. I performed *in vitro* translation assays using 2 pmol ribosomes, testing three independently isolated ribosome samples for each purification condition in technical duplicate (**Figure 10**). I found that technical replicates yielded very similar results. The ribosomes isolated from cells lysed by French press were most similar to each other. Finally, the signal from ribosomes isolated from cells lysed by French press was significantly higher on average than that of ribosomes isolated after lysis by BugBuster®. We can conclude that using 2 pmol LVS ribosomes in the *in vitro* translation reaction yields measurable and consistent results across most purifications and that ribosomes purified from French press-lysed cells are more active at low concentrations than those purified from BugBuster®-lysed cells.

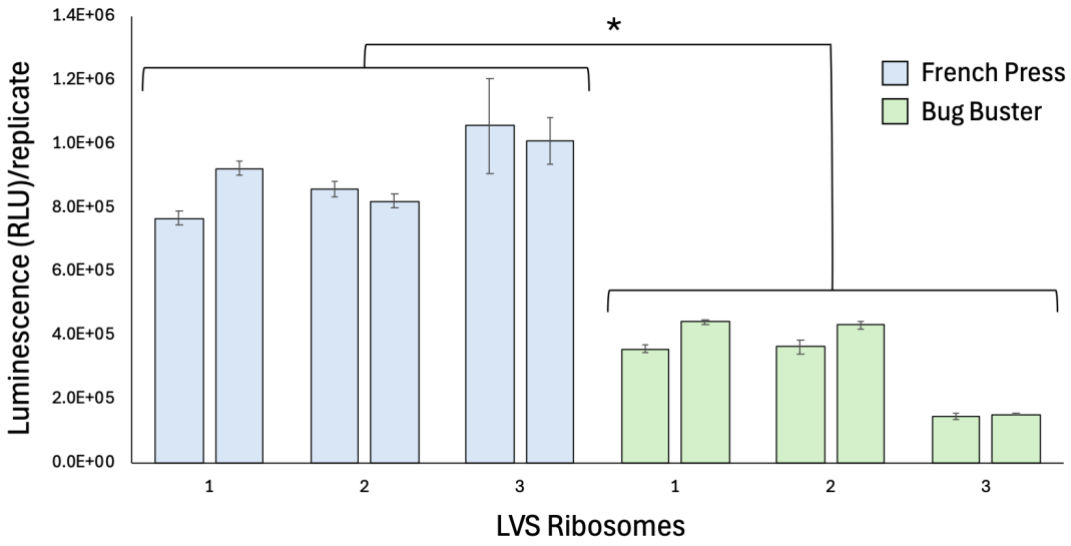

Figure 10. *F. tularensis* LVS ribosomes purified from French press-lysed cells are more active than those purified from BugBuster®-lysed cells. Chart shows the results from *in vitro* assays using pKR214 as a template and 2 pmol LVS ribosomes from different purifications. Each number represents a different ribosome purification, with bars above each number representing technical replicates. Error bars represent 1 SD. Brackets above bars indicate statistical comparison among groups by t-test. Asterisk indicates comparison between two sets of ribosomes. *$P < 0.05$ by *t*-test.

Lysis method affects processing of ribosomal RNA

To further understand the differences between ribosomes isolated from cells lysed by French press or BugBuster®, I extracted RNA from several 70S ribosome samples and analyzed the rRNA by agarose gel electrophoresis. In particular, I compared 70S ribosomes purified from cells lysed by French press to those purified from cells lysed using BugBuster®, as well as total RNA purified using a formamide-based extraction method (RNA-Snap) and total RNA isolated after cell lysis with BugBuster® (**Figure 11**). I found that in all the samples using BugBuster® for lysis, there was no band corresponding to the 23S rRNA but rather several smaller bands beneath the band for 16S rRNA, indicating that the 23S rRNA was being processed or fragmented

during some stage of the purification after lysis with BugBuster®. This contrasted with the analysis of ribosomes isolated from cells lysed by French press, which have a prominent 23S rRNA band. This discovery led me to discontinue my use of BugBuster® chemical lysis as a method for lysing cells prior to purifying ribosomes, but it did yield some useful information. Specifically, I found that active ribosomes can be purified from cells after lysis with BugBuster® detergent, but rRNA fragmentation or processing occurs, possibly the result of RNase activity within the proprietary chemical mixture.

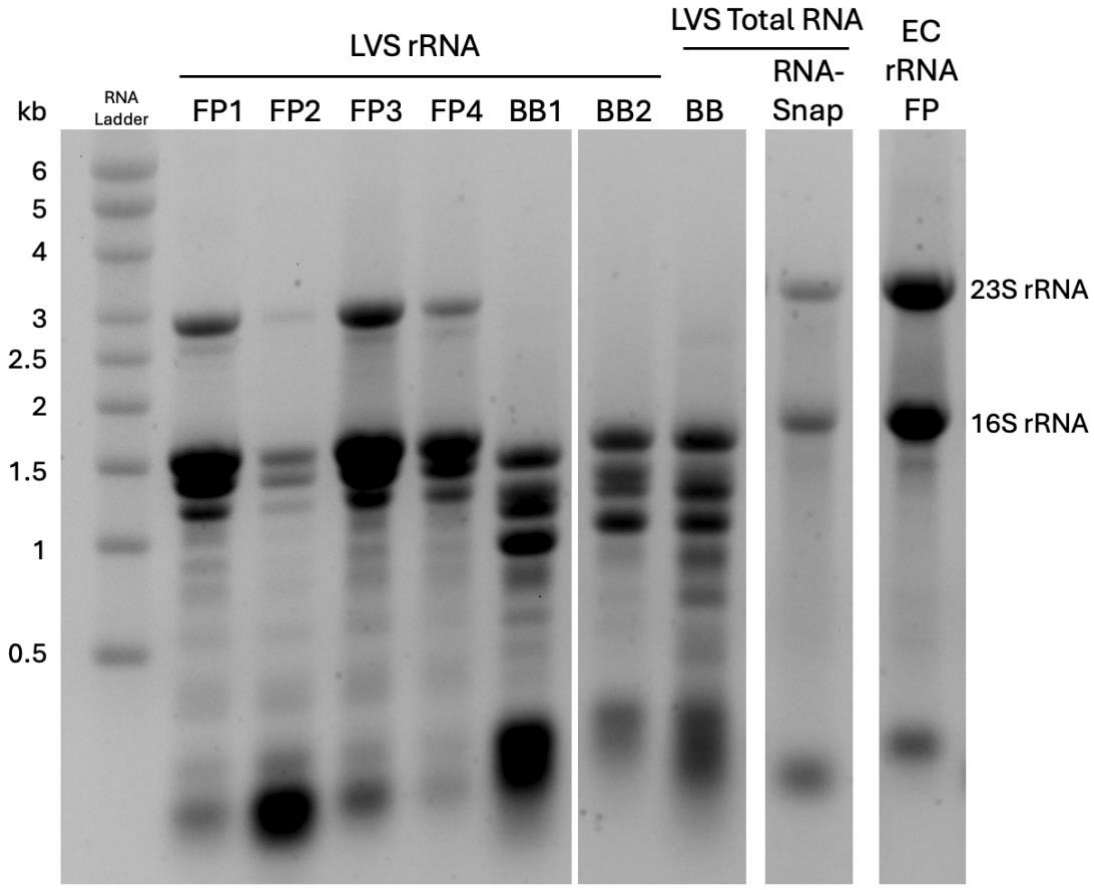

Figure 11. *F. tularensis* LVS ribosomes purified from BugBuster®-lysed cells have fragmented 23S rRNA. Agarose gel electrophoresis of indicated RNA (total RNA extracted from cells or rRNA extracted from 70S ribosomes) denatured by glyoxal treatment. FP indicates a French press was used for cell

lysis. BB indicates BugBuster® was used for cell lysis. Note that gel image was cropped and re-ordered for clarity. LVS: *F. tularensis* LVS; EC: *E. coli*.

Reproducible *in vitro* translation by *F. tularensis* LVS ribosomes

The primary goal of my work was to develop a reproducible assay to measure translation by *F. tularensis* ribosomes. Having optimized purification and *in vitro* assay conditions, I compared the luminescence data from several assays performed on different days using these optimized conditions (2-2.5 pmol ribosomes from French press-lysed *F. tularensis* LVS; **Figure 12**). The luminescence values appear to be reproducible across different purifications, biological replicates, and technical replicates when the assay includes about 2 pmol *F. tularensis* LVS ribosomes. The signal (~1x10^6 RLU/replicate) is also adequate compared to previous reactions with 40 pmol *F. tularensis* LVS ribosomes (~1.3-2.5x10^6 RLU/replicate; **Figure 6**). Specifically, we obtained about one-third of the signal for one-twentieth of the ribosome concentration. This indicates that 2 pmol may be an ideal amount of ribosomes to use in the *in vitro* assay while maintaining reproducibility and adequate signal strength.

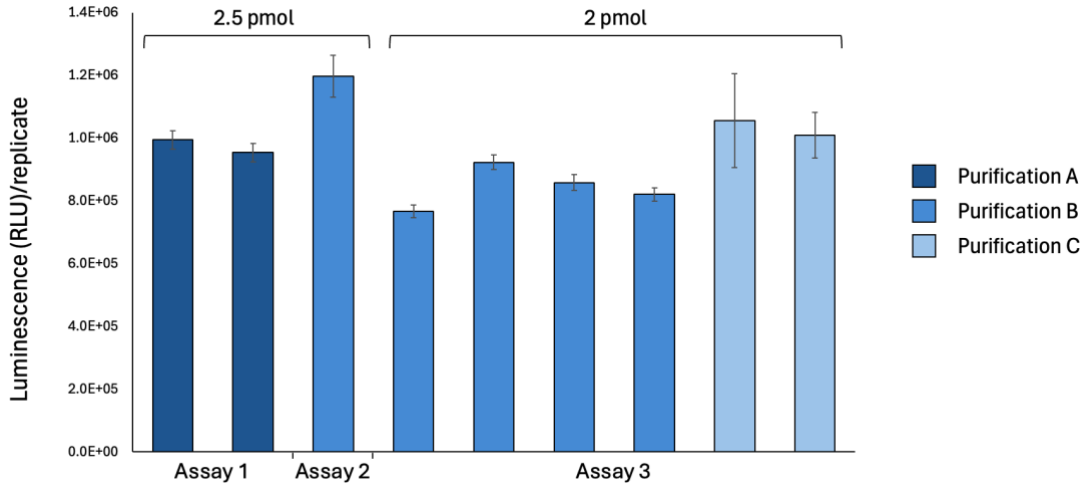

Figure 12. Comparison of luminescence data for several assays shows reproducible translation signal with 2 or 2.5 pmol *F. tularensis* LVS ribosomes. Chart shows the results from *in vitro* assays using pKR214 as a template and indicated amount of LVS ribosomes from different purifications. Salt-washed *F. tularensis* LVS ribosomes were purified from French press-lysed cells. Experiments were performed in technical triplicate. Error bars represent 1 SD.

CHAPTER 4

CONCLUSIONS

Summary

This research project is part of a larger effort to identify ways in which the ribosomal protein, bS21, regulates translation of mRNAs with specific leader sequences in *Francisella tularensis*. Previous studies using reporter assays in bacterial cells (*in vivo*) made significant progress towards identifying leader sequences that confer regulation by bS21, but we would like to determine if these results can be recapitulated using purified ribosomes (*in vitro*). Once a working *in vitro* system has been established, we can build upon experimental data from *in vivo* studies and develop a more comprehensive model of regulation by bS21. The primary goal of this research project was to develop an *in vitro* translation assay using *F. tularensis* ribosomes with the NEB PURExpress® *in vitro* translation kit. To achieve this goal, I developed a sensitive and easily modifiable reporter construct, purified active ribosomes from *E. coli* and *F. tularensis* LVS, and optimized the reproducibility of the *in vitro* assay.

Using standard cloning techniques, I generated a reporter construct for the *in vitro* translation assay that is sensitive and easy to modify. I also purified salt-washed *E. coli* MRE600 ribosomes and determined that they were translationally active in the *in vitro* assay. Then, I replicated these results using ribosomes from multiple purification attempts, validating that we can reproducibly isolate active ribosomes. In addition, I demonstrated that *E. coli*

ribosomes are inhibited by kasugamycin and thiostrepton, indicating that we can use the assay to measure translation inhibition by antibiotics. I found that *F. tularensis* ribosomes appear to aggregate or clump more readily than *E. coli* ribosomes, but that when there are fewer ribosomes in a sample, there is less aggregation. This aggregation appears to impact translational activity, as I found that luminescence per pmol of ribosomes increases as ribosome concentration decreases. After further testing, I concluded that using 2 pmol of *F. tularensis* LVS ribosomes in the *in vitro* assay yields a measurable and consistent signal across multiple ribosome purifications. This optimized *in vitro* assay that I developed can be used in future research to easily measure translation efficiency of mRNAs with different leader sequences and will contribute to a better understanding of how bS21 homologs regulate translation in *F. tularensis.*

Successful development of a reporter plasmid for *in vitro* translation

I generated a plasmid template for *in vitro* translation reactions which uses the T7 promoter to drive expression of nLuc with the 5′ untranslated region (UTR) of *tul4* (pKR214). This reporter construct allows for increased sensitivity compared to fluorescent reporters, as the luminescent signal resulting from nLuc activity was consistently higher than the signal from other tested reporters. It is easy to modify, because the UTR and promoter region are flanked by DNA encoding two enzyme digestion sites (NotI and XmaI). This allows for the removal of this region and its replacement with a DNA fragment containing specific modifications to the leader sequence, providing us with a reporter construct that can easily measure the translation efficiency of various UTRs. In

the future, we can modify this plasmid to test UTRs from virulence genes that are regulated by bS21-2 to determine if this regulation is direct, and to test for specific sequence motifs that impact regulation.

Reproducible purification of active ribosomes from *E. coli*

I found that the salt-washed *E. coli* MRE600 ribosomes I purified were as active as kit-provided ribosomes in *in vitro* translation reactions and were within a log-fold difference in signal strength across purifications and *in vitro* assays. These tests validated that we can reproducibly isolate active salt-washed ribosomes using our sucrose cushion ribosome isolation protocol.

***In vitro* assay measures translation inhibition by antibiotics**

Using the *in vitro* system, I discovered that kasugamycin and thiostrepton significantly inhibit translation with *E. coli* ribosomes at a concentration of 1mM. This finding indicates that we can measure translation inhibition by antibiotics in our *in vitro* assay. In the future, this assay will allow us to test the ability of other antibiotics to inhibit translation or to identify whether potential antibiotic compounds act by inhibiting ribosome activity.

Optimization of growth conditions for *F. tularensis* results in greater ribosome yield

I optimized *F. tularensis* LVS growth conditions to increase cell and ribosome yield, which allows us to purify a larger quantity of *F. tularensis* ribosomes for *in vitro* assays and to conduct more assays with the same samples.

Ribosome aggregation observed in sucrose gradients

I analyzed sucrose gradient profiles from various ribosome samples and determined that in our *F. tularensis* ribosome purifications, we have active 70S ribosomes along with either 70S aggregates or 100S ribosomes, suggesting that in each sample there may be a subset of ribosomes that are inactive. This was unexpected, because similar amounts of *E. coli* ribosomes did not show evidence of aggregation or clumping in sucrose gradients. This discovery opens the possibility that similar aggregation may occur with purified ribosomes from other bacterial species. Future research studies using ribosomes purified from species other than *E. coli* could check for aggregation by running purified ribosome samples on sucrose gradients.

I observed a correlation between the ribosome concentration in a sample and the amount of aggregation in the gradient profile. Specifically, when there were fewer ribosomes in a sample, there was less aggregation. Future studies could test modifications to the ribosome purification protocol and experiment with different buffer compositions to limit *F. tularensis* ribosome aggregation and produce homogenous ribosome samples.

Use of BugBuster® as a lysis agent leads to rRNA fragmentation or processing

I found that the 23S rRNA purified from cells lysed using the proprietary detergent BugBuster® was either fragmented or processed. Our goal is to study translation in unprocessed ribosomes, ruling out the use of BugBuster® to chemically lyse cells when purifying ribosomes. The French press uses high pressure to physically shear open cells and is commonly used as a method of

cell lysis for purification of active ribosomes (Mehta et al. 2012). In the future, the Ramsey lab will continue to use the French press to lyse cells. Other forms of chemical lysis could also be investigated, such as the commercially available lysis buffer, B-PER (Thermo Scientific; Mehta et al. 2012).

Reproducible *in vitro* translation by *F. tularensis* LVS ribosomes

I observed that the signal per pmol of ribosomes increased as ribosome concentration decreased, with the highest per-ribosome signal coming from reactions containing 2 pmol or fewer ribosomes from *E. coli* or *F. tularensis*. Using 2 pmol of *F. tularensis* LVS ribosomes *in vitro* yielded measurable and consistent results across multiple ribosome purifications and may be an ideal amount of ribosomes to use while ensuring a strong and reproducible signal in the *in vitro* assay.

Future Directions

Having optimized our *in vitro* assay, we can now consider additional research questions. Future studies could investigate why fewer ribosomes lead to more signal in the *in vitro* assay. It seems that aggregation plays a role, but other factors may also be at work. For example, higher ribosome concentrations in a sample might cause all the available mRNA to be bound by ribosomes, leading to a decrease in signal per ribosome at higher concentrations. Furthermore, any inactive or “dead” ribosomes bound to mRNA transcripts would decrease the translation efficiency within a sample. Preliminary results suggest that the decline in translation efficiency at higher ribosome concentrations may be reporter specific—in one experiment using an sfGFP

reporter, the same consistent increase in signal per ribosome at lower concentrations was not observed. Additionally, future experiments could explore how ribosome concentration or buffer composition impacts nLuc translation or activity.

We could also investigate factors that contribute to rRNA processing in BugBuster®-lysed cells by using RNA-seq to analyze ribosome samples. After extracting RNA from purified ribosomes or whole cell lysates, we could copy the rRNA to make complementary DNA (cDNA) and sequence the cDNA using next-generation sequencing techniques. This data would help us identify where the 23S rRNA is being fragmented and may help us understand any RNA processing events that are taking place in these samples.

A functional *in vitro* translation assay will allow us to measure the translation efficiency of mRNAs and identify what leader sequences lead to preferential translation by ribosomes with bS21-2. We can test leader sequences from *mraY*, *iglA*, *rpsU2*, *yqeY*, and other genes identified as being regulated by bS21-2 *in vivo* (Trautmann and Ramsey 2022) to better understand whether this regulation happens directly. Since the *in vitro* assay contains only the factors necessary for transcription and translation, we can more easily determine whether bS21-2 directly regulates the translation of particular mRNAs without having to consider the many interactions taking place within live cells. We can also test modified UTRs with ideal Shine-Dalgarno sequences or other motifs identified as contributing to regulation by bS21-2 (Trautmann et al. 2023) to see how they impact regulation. Such experiments would help us characterize more

precisely the interactions between bS21-2 and the mRNA that are necessary for regulation to occur. In addition, we can use the assay to compare the translation efficiency of ribosomes with different bS21 homologs. Because the *F. tularensis* genome encodes three homologs of the bS21 protein, it is possible that each homolog may regulate a certain subset of mRNAs. This assay would allow us to test this possibility by measuring the translation efficiency of mRNAs with ribosomes containing specific bS21 homologs.

Finally, with this assay, we can measure translation inhibition by antibiotics. In addition to testing for translation inhibition with ribosomes from a particular species, we could test ribosomes from the same species with different composition (e.g., containing different ribosomal protein homologs). Such investigations would shed light on antibiotic mechanisms of action and provide insight into methods of antibiotic resistance. The assay could be further used to test compounds that may inhibit ribosomes, allowing validation of potential ribosome-targeting antibiotics and informing new approaches to antibiotic development.

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