EXAMINING THE REGULATION OF THE BS21 HOMOLOGS IN *FRANCISELLA TULARENSIS*

BY

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ABSTRACT

Francisella tularensis is a facultative intracellular pathogen, which relies on a network of regulation for its virulence. It contains three homologs of the ribosomal protein bS21. These three ribosomal protein homologs can independently associate with ribosomes, leading to ribosomal heterogeneity. The second homolog, bS21-2, is implicated in the positive regulation of a number of virulence proteins. Additionally, transcriptomic data indicates that bS21-2 is autoregulated. The mechanism by which this autoregulation occurs is unknown, as is the regulation of the other two homologs, bS21-1 and bS21-3. In the following chapters, I will show that bS21-1 and bS21-3 do not autoregulate their transcript. Further, I will demonstrate that the leader sequence of bS21-2 is sufficient to result in autoregulation of its transcript. And in particular, a specific portion of the leader sequence is sufficient for regulation. This regulation is potentially accomplished through the degradation of the bS21-2 transcript. Alternatively, I will show that the bS21-2 protein is not autoregulated.

I will then explore different environmental conditions to determine what conditions may up-regulate bS21-1 and bS21-3. Particularly, growth on CHA-H results in up-regulation of transcript abundance of bS21-1 and bS21-3, as compared to supplemented MHB. This growth condition leads to a subsequent increase of both bS21-1 and bS21-3 protein. In addition, low pH increases bS21-1 transcript abundance, but the effect on protein is unclear.

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PREFACE

This thesis has been prepared in manuscript format according to the guidelines of the Graduate School of the University of Rhode Island. The thesis includes an introductory chapter, two manuscripts, and a summary chapter:

Chapter 1: Literature Review and Introduction

Chapter 2: “Control of bS21 homolog production in *Francisella tularensis*” was prepared for submission to the Journal of Bacteriology.

Chapter 3: “Identification of conditions that lead to changes in bS21-1 and bS21-3 abundance in *Francisella tularensis*” was prepared for submission to the Journal of Bacteriology.

Chapter 4: Conclusions and Further Questions

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CHAPTER 1

REVIEW OF THE LITERATURE

Francisella tularensis is an intracellular pathogen.

Francisella tularensis is a Gram-negative coccobacillus bacterium. It is the causative agent of tularemia, also known as rabbit fever (Keim, Johansson, and Wagner 2007). It is a facultative intracellular pathogen and must grow within macrophage to cause illness. Due to its high infectivity and low infectious dose (estimated at 10 cells), it is classified as a potential bioweapon alongside other bacterial species such as *Bacillus anthracis*, the causative agent of anthrax (Dennis et al. 2001). That said, it is an environmental pathogen that is endemic to North America, Europe, and Asia. The majority of reported cases arise from the United States, with the highest incidence in the south-central region of the country (Keim et al. 2007).

There are multiple routes by which *F. tularensis* may infect a host, including inhalation, ingestion, or through contact with open wounds (Keim et al. 2007). The disease tularemia may present in a number of ways, which vary dependent on the route of entry. This presentation can include flu-like symptoms such as malaise and cough. It may also cause ulcers or swelling in areas such as the stomach, mouth, or eyes (Keim et al. 2007).

The two subspecies of *F. tularensis* that are the most clinically relevant are *F. tularensis* subsp. *tularensis* and *F. tularensis* subsp. *holarctica* (Kingry and Petersen 2014; Olsufjev and Meshcheryakova, 1983; Petersen and Molins 2010). Infection with *F. tularensis* subsp. *tularensis* causes the most severe

human disease and can be lethal (Kingry and Petersen 2014). In contrast, infection by *F. tularensis* subsp. *holarctica* generally leads to less severe disease outcomes (Kingry and Petersen 2014).

Three strains of *F. tularensis* are commonly used in laboratory research. They are the highly pathogenic *F. tularensis* subsp. *tularensis* SCHU S4; *F. tularensis* subsp. *holarctica* LVS (live vaccine strain), which is a strain attenuated for virulence in humans; and a strain from related species that is not infectious to humans, *Francisella novicida* U112.

F. tularensis subsp. *tularensis* SCHU S4 is highly virulent strain derived from a clinical sample from an infected finger (Eigelsbach, Braun, and Herring 1951; Hesselbrock and Foshay 1945). Like all *F. tularensis* species, its genome contains two copies of the Francisella Pathogenicity Island (FPI), which encodes a type VI secretion system (T6SS) that is critical for virulence (Bell, Owen, and Larson 1955). In addition, the SCHU S4 genome was the first *F. tularensis* strain to be fully sequenced. While it is highly pathogenic, making it an ideal model for research regarding *F. tularensis* virulence, it is also classified as a select agent. Thus, in order to prevent laboratory-acquired infections, BSL-3 safety measures must be used in order to study it safely.

Unlike SCHU S4, *F. novicida* U112 does not cause human disease and is only pathogenic in animal hosts. Unlike *F. tularensis* species, U112 has a single copy of the FPI, making it a tractable model system for genetic studies of the FPI. However, it is not an ideal model for human virulence, as it does not infect immunocompetent individuals (Kingry and Petersen 2014; Nano et al. 2004).

F. tularensis subsp. *holarctica* LVS is a strain which is attenuated for virulence in humans and was originally developed as a vaccine. Importantly, it has the ability to infect macrophage and cause disease in animal models, but can be safely used in BSL2 laboratory settings. Despite attenuation, it remains highly related to similar virulent strains; the LVS genome has mutations in 35 protein coding regions compared to a pathogenic *F. tularensis* subsp. *holarctica* strain, FSC200. The majority of these differences are single nucleotide polymorphisms, SNPs, leading to loss or impaired function of 15 proteins (Rohmer et al. 2006). One gene in particular, *pilA*, a putative Type IV pilin, is mutated in LVS. Re-introduction of this functional gene restored LVS virulence to levels comparable to non-attenuated *F. tularensis* subsp. *holarctica* strains (Salomonsson et al. 2009). Additionally, a gene important for iron uptake in pathogenic strains (*fupA*) has recombined with a neighboring gene (*fupB*) in LVS, resulting in less efficient ferric iron transport and reduced virulence (Ramakrishnan, Sen, and Johnson 2012; Salomonsson et al. 2009). Together, the reduced biosafety level needs and the ability of LVS to infect animal models make the LVS strain an ideal candidate to study mechanisms of pathogenicity in a biologically relevant manner.

There are a number of genes important for virulence in *F. tularensis* (Jones et al. 2014; Rowe and Huntley 2015). Many factors have been linked to regulation of virulence genes in *F. tularensis*, including temperature, iron availability, oxidative stress, host-cell-specific components, transcriptional regulators, and translational regulators (Dai et al. 2011; Trautmann and Ramsey

2022). Arguably, the virulence factor with the most well-understood regulation in *F. tularensis* is the Francisella Pathogenicity Island (FPI), so in the following section it will provide an example of how virulence genes are controlled in this pathogen.

Francisella Pathogenicity Island (FPI)

The FPI is a cluster of 16 conserved genes with three variable genes encoded by Francisella genomes (de Bruin et al. 2011; Nano et al. 2004; Nano and Schmerk 2007). The FPI contains the genes encoding T6SS proteins. These genes include structural components of the T6SS and putative effectors (Brodmann et al. 2017; Clemens, Lee, and Horwitz 2018; Eshraghi et al. 2016; Lai, Golovliov, and Sjöstedt 2004) (Figure 1). The T6SS is essential for intracellular survival and virulence, as it is necessary for phagosomal escape (Brodmann et al. 2017).

Consistent with their essentiality for intramacrophage survival, FPI genes are up-regulated when *F. tularensis* infect macrophage (Bent et al. 2013; Golovliov et al. 1997). Significant work has revealed how the FPI genes are controlled at the level of transcription. Specifically, the FPI genes are regulated by the transcription factors MglA, SspA, and PigR, acting together with the small molecule ppGpp. The specific mechanism of regulation relies on the intrinsic formation of the MglA-SspA complex, and its interactions with the RNA polymerase. In the presence of ppGpp, PigR (also referred to as FevR in *F. novicida*) interacts directly with the MglA-SspA complex to positively regulate genes with a specific PigR-responsive element (PRE), including FPI genes

(Charity et al. 2007; Cuthbert et al. 2017; Ramsey et al. 2015; Rohlfing and Dove 2014; Travis et al. 2021).

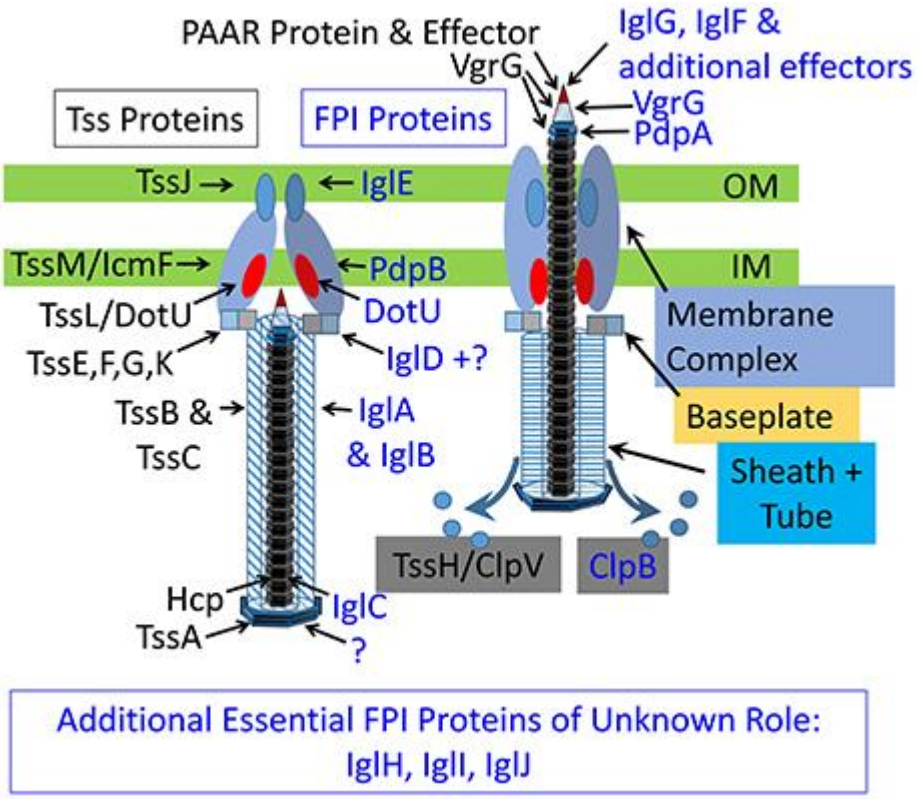

Figure 1. A number of Type VI Secretion System proteins and their functions are defined in *F. tularensis.* Illustration depicting the extended (left) and contracted (right), forms of the canonical and *Francisella* Type VI Secretion System (T6SS). Identified *F. tularensis* T6SS proteins are indicated in blue font, whereas canonical elements are indicated in black font (Clemens et al. 2018).

Because ppGpp influences the activity of PigR-regulated genes like the FPI, factors that influence ppGpp levels influence FPI gene expression. That said, a number of genes influence ppGpp levels (Buchan et al. 2009; Faron et al. 2013; Travis and Schumacher 2022). Ultimately, this leads to a complex network of regulators all affecting *F. tularensis* pathogenicity.

Ribosome production is tightly regulated.

Ribosomes are large, multi-component molecular machines, composed of both ribosomal RNAs (rRNAs) and ribosomal proteins (r-proteins). They act as a key component of cellular life, translating mRNA into protein. Ribosomes have a large number of components, and are energetically costly to the cell. Production of ribosomal components is tightly controlled so that each component is produced within stoichiometric ratio. Studies have elucidated multiple mechanisms by which individual rRNAs and r-proteins are regulated to retain their stoichiometric balance.

As rRNA is the primary component of the ribosome, its regulation has been a particular focus of study. Production of rRNA is regulated at the transcriptional level and depends on factors such as the growth state of the cell. In particular, the promoter of rRNA plays a significant role in the control of rRNA. These promoters are quite strong and promoter activity is carefully regulated through multiple inputs such as the small molecule ppGpp, abundance of the initiating NTP of transcription, and DksA, a component of the transcription machinery (Paul et al. 2004).

Bacteria may encode more than 50 r-proteins, 34 of which are universally conserved (Yutin et al. 2012). Regulation of r-protein production can be complex and can occur at multiple levels. Regulation of r-proteins can start at the level of DNA organization, as operons encoding r-proteins are often organized to allow for co-regulation of multiple r-protein-coding genes.

Additionally, r-proteins may act as regulators of their own transcript. Specifically, some r-proteins can bind their own transcript in addition to their

ability to bind ribosomal RNA, particularly if that r-protein is in excess compared to the amount of r-protein needed for ribosome assembly. Attenuation and destabilization of mRNA transcripts are major pathways in which an r-protein may control translation of its own transcript. Attenuation describes the early termination of an r-protein transcript via the r-protein, directly or indirectly. It can either be Rho-dependent or Rho-independent. Regardless of the necessity of Rho, this type of regulation relies on the formation of specific secondary structures of the mRNA transcript that lead to termination (Turnbough 2019). Alternatively, r-proteins may recruit nucleases to their own transcripts, leading to the destabilization of their mRNA post-transcriptionally. As with attenuation, the secondary structures of the mRNA transcript are often important for degradation (Rauhut and Klug 1999).

Finally, r-proteins may regulate their own production at the level of translation, by inhibiting translation initiation for its own transcript. There are several mechanisms by which this could occur. One possibility is that direct contact by the r-protein with the Shine-Dalgarno of its transcript physically prevents the transcript from becoming associated with ribosomes. Alternatively, direct binding of the r-protein could cause conformational changes in the mRNA to prevent translation, or the r-protein may interrupt a step in the translation initiation pathway (Zengel and Lindahl 1994).

Production of one r-protein can be controlled by multiple mechanisms. The next section will illustrate this by describing regulation of ribosomal protein uL10 (previously referred to as L10; Ban et al. 2014).

uL10 Regulation

uL10, the bacterial ribosomal protein encoded by *rplJ*, is a large subunit ribosomal protein. In the ribosome structure, uL10 forms the stalk complex with bL12, performing the essential function of facilitating interactions between GTP-bound translation factors and the ribosome (Diaconu et al. 2005) The *rplJ* gene is found in an operon upstream of *rplL* (bL12 or, when acetylated, bL7), denoted *rplJL* (O'Connell and Thomashow 2000). These r-proteins are followed by *rpoB* and *rpoC* encoding the *β* and *β′* subunits of RNA polymerase (Fukuda 1980). uL10, along with bL12, binds to the 23s rRNA early in ribosomal assembly (de Narvaez and Schaup 1979).

In *Escherichia coli*, the first level of regulation of uL10 is at the transcriptional level. A study determined the promoter strength of *rplJ* in comparison to other gene promoters, and found that the strength of the promoter for transcription changed with temperature and salt concentrations (Kajitani and Ishihama 1983). Further, the *rplJ* gene is co-transcribed with both *rpoB* and *rpoC* (Linn and Scaife 1978; Yamamoto and Nomura 1978). While promoter strength and environmental conditions can lead to the transcriptional regulation of uL10, proposed intrinsic attenuators in the operon reduce the expression of the downstream *rpo* genes. This results in approximately 5 times the amount of mRNA encoding the ribosomal proteins compared to the RNA polymerase proteins (Barry, Squires, and Squires 1979).

When uL10 is present in excess of the amount needed for ribosome assembly, a negative feedback mechanism (autoregulation) leads to its

repression. This requires uL10 and the uL10-L7/L12 complex (Fukuda 1980). This autoregulation was determined to act at a specific site, near the translational start site of uL10, in particular at a bulge loop secondary structure of the mRNA where it is proposed the complex binds and changes conformation (Climie and Friesen 1987; Yates et al. 1981). Additionally, it is suggested that uL10 when not coupled with L7/L12, is subject to proteolytic decay (Petersen 1990).

While the regulation of uL10 is both interesting and complex, it is important to note that it does not set a canonic precedent for each ribosomal protein. Some others share the same regulatory mechanism, however, there is a variety of mechanisms described in other literature (Zengel and Lindahl 1994). It is possible that there are more mechanisms in the control of r-proteins which are not yet elucidated.

Ribosomes are heterogenous.

The ribosome is generally thought to operate as a homogenous machine, indiscriminately translating mRNAs. However, ribosome composition can be heterogenous with respect to its rRNAs and proteins, meaning that all ribosomes in an organism, or even in a single cell, may not have precisely the same composition (Byrgazov, Vesper, and Moll 2013). There are a number of sources of genetically-encoded ribosome heterogeneity; including multiple rRNA operons, post-transcriptional modifications of rRNA, post-translational modifications of r-proteins, or incorporation of one of multiple homologs of a given r-protein. Ribosome heterogeneity has been identified in multiple

organisms, including well-studied organisms such as *E. coli*. In the case of ribosomal heterogeneity induced by zinc concentration, the regulation of ribosomal composition is well-studied.

Zinc as a regulator of ribosomal composition

In some bacterial species, such as *Mycobacterium tuberculosis, Mycobacterium smegmatis, Bacillus subtilis,* and *E. coli*, there are paralogs of r-proteins which either coordinate or do not coordinate zinc. When in zinc-rich conditions, these organisms will produce the zinc-coordinating paralog. However, decreasing zinc concentrations leads to production of the alternate paralogs of these r-proteins, which do not coordinate zinc (Dow et al. 2021; Dow and Prisic 2018; Li et al. 2020; Nanamiya et al. 2004). The alternate paralogs are typically regulated transcriptionally by Zur, the zinc uptake regulator protein. Specifically, Zur, when bound by zinc, will repress expression of certain transcripts by binding a so-called Zur box in their promoter regions, including in the promoter regions of genes encoding the alternative r-proteins. When zinc concentrations decrease, Zur does not coordinate zinc and does not bind DNA, thus repression is lifted (Akanuma et al. 2006; Dow et al. 2021; Dow and Prisic 2018; Li et al. 2020; Nanamiya et al. 2004; Nanamiya, Kawamura, and Kosono 2006; Nanamiya and Kawamura 2010; Prisic et al. 2015; Rasmussen et al. 2022; Shin and Helmann 2016). De-repression of the genes encoding alternate r-proteins provides a straightforward mechanism to permit their production under low zinc concentrations.

In *B. subtilis*, switching the zinc-coordinating bL31 paralog RpmE in the ribosome for the alternate paralog, YtiA, allows for the release of the zinc coordinated by RpmE when environmental zinc is low (Nanamiya and Kawamura 2010; Shin and Helmann 2016). This suggests that this ribosomal state switching is useful in modulating zinc availability when environmental reservoirs are low.

Beyond regulating zinc availability, how incorporation of non-zinc coordinating paralogs impacts the function of ribosomes is not well-understand. In *E. coli*, one paralog of r-protein bL31, bL31A, which coordinates zinc, appears to lead to higher fitness under low temperatures and alters translation reading frame maintenance (Lilleorg et al. 2020). In *M. tuberculosis* ribosomal switching of S18 paralogs may facilitate the transition from intracellular to extracellular environments, wherein zinc concentrations are lower due to host immune responses. The production of a non-zinc coordinating homolog would necessarily allow translation to continue in these zinc-deplete environments (Prisic et al. 2015). Further, in *M. smegmatis*, alternative ribosomes have altered translation activity, leading to the possibility that alternative ribosomes regulate proteome composition (Chen et al. 2020).

Specialized ribosomes

The presence of ribosome heterogeneity raises the possibility that ribosomes with different compositions have altered function. Ribosomes with altered composition and function are referred to as “specialized ribosomes.” It

is proposed that specialized ribosomes may function as regulators of gene expression (Byrgazov et al. 2013).

bS21 leads to ribosomal heterogeneity in *F. tularensis.*

The ribosomal protein bS21

bS21 is a small subunit ribosomal protein encoded by the gene *rpsU*. *rpsU* is often, though not always, encoded on the so-called macromolecular synthesis (MMS) operon. This operon is conserved across diverse species, including many Gram-negative species (Versalovic et al. 1993). The MMS operon is typically structured as *rpsU-dnaG-rpoD*. The *rpsU* gene encodes bS21, which has been implicated in translation initiation (Chang and Craven 1977; Van Duin and Robert 1981). The *dnaG* gene encodes DNA primase, which is critical for initiation of DNA replication (Rowen and Kornberg 1978). The *rpoD* gene encodes σ^{70}, which is the primary sigma factor responsible for transcription of many housekeeping genes (Miura et al. 2015). Importantly, each of these three genes is important for the initiation of different steps of macromolecular synthesis, thus leading to the common name of the operon.

As indicated, bS21 has been implicated in translation initiation. Specifically, one study deactivated mRNA binding activity of the ribosome by inducing ribosomal protein damage, preventing mRNA binding. By adding back undamaged proteins, the researchers determined that bS21 was one of the proteins required to allow the ribosome to bind mRNA again. This indicated that it, along with some other proteins, were critical in the initiation of translation (Chang and Craven 1977). Additionally, it was determined that bS21 is required

to translate certain mRNAs (Van Duin and Robert 1981). However, bS21 is thought to be non-essential, as it is not encoded in all bacteria (Galperin et al. 2021), although it is encoded by some phage (Mizuno et al. 2019).

In bacteria that do encode bS21, there are specific phenotypes observed when it is lost. For example, in *B. subtilis*, deletion of *rpsU* lead to filamentous cells with impaired motility (Akanuma et al. 2012). In *Staphylococcus aureus*, clinical samples with increased resistance to daptomycin and vancomycin have mutations in *rpsU* (Basco et al. 2019; Blake and O'Neill 2013; Friedman, Alder, and Silverman 2006). In *Burkholderia pseudomallei*, a Tn-Seq strategy using a transposon mutant library passaged through a mouse infection model identified bS21 as critical for *B. pseudonallei* to cause disease (Gutierrez, Yoder-Himes, and Warawa 2015). Through a similar method, a bS21 homolog in *F. tularensis*, *rpsU1*, was implicated as a potential virulence gene in mice (Su et al. 2007).

bS21-2 in F. tularensis

The genome of *F. tularensis* encodes three distinct bS21 homologs, bS21-1, bS21-2, and bS21-3 (encoded by *rpsU1, rpsU2,* and *rpsU3*, respectively). This is notable due to the limited size of the *F. tularensis* genome (approximately 2 Mbp). Small, reduced genomes are characteristic of intracellular pathogens, making the presence of three bS21 homologs in *F. tularensis* remarkable (Larsson et al. 2005; Murray et al. 2021; Riffaud, Rucks, and Ouellette 2023).

The most abundant bS21 homolog in *F. tularensis* is bS21-2 (Trautmann and Ramsey 2022). This homolog is encoded in the *F. tularensis* MMS operon,

which includes an additional gene between *rpsU* and *dnaG*, *yqeY* (Figure 2). The protein YqeY is thought to aid in accurately charging tRNAs (Deniziak et al., 2007). Loss of bS21-2 leads to an intramacrophage growth defect and is implicated in control of translation for certain proteins (Trautmann and Ramsey 2022).

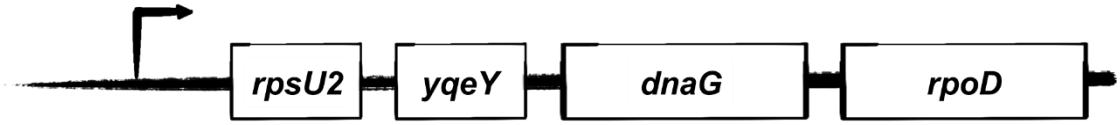

Figure 2. The *rpsU2* operon structure. The sequence of genes of the macromolecular synthesis (MMS) operon of *F. tularensis* (not to scale).

The role of bS21-2 in F. tularensis

A recent study by the Ramsey lab compared wild-type *F. tularensis* with cells lacking bS21-2 and analyzed differences in both protein and transcript abundance. This revealed that loss of bS21-2 leads to changes in protein abundance for 162 genes that cannot be explained by a concordant change in transcript abundance (Trautmann and Ramsey 2022) (Figure 3). These genes include a number of critical virulence genes. Further, the study revealed that cells lacking bS21-2 have a reduced ability to grow within macrophage. The data from this study is consistent with a model in which bS21-2 positively influences translation of virulence proteins (Trautmann and Ramsey 2022).

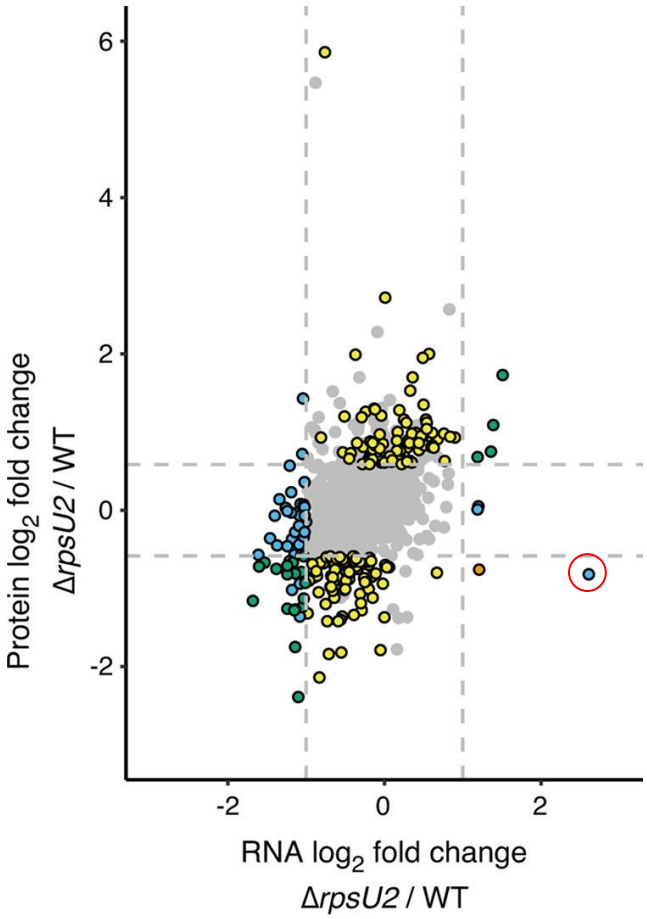

Figure 3. Cells lacking bS21-2 have changes in protein abundance that cannot be explained by changes in transcript abundance. Dots indicate genes. Log_2-transformed fold change of transcript abundances (x-axis) versus log_2-transformed fold change of protein abundances (y-axis) between wild-type and Δ*rpsU2* cells. The dot representing the gene *yqeY* is indicated in a red circle (Trautmann and Ramsey 2022).

We propose a general model in which each bS21 homolog is able to independently associate with ribosomes, and subsequently preferentially initiate translation of certain transcripts. With regard to bS21-2, we have determined that bS21-2 affects virulence protein abundance. However, the mechanism by which bS21-2 exerts its effects on the proteome of *F. tularensis* is an ongoing research topic. We have determined that specific leader sequences do lead to

control by bS21-2, and that this control depends on an imperfect Shine-Dalgarno sequence (Trautmann et al. 2023).

Ribosome heterogeneity in F. tularensis

While zinc is known to affect paralogous ribosomal protein abundances resulting in changes in ribosome composition, none of the three homologs of bS21 in *F. tularensis* coordinate zinc. Further, the regulatory network controlling production of these three homologs has not been previously investigated. Transcriptomic data of wild-type cells, cells lacking bS21-2, and cells lacking bS21-2 with ectopic expression of each of these three homologs reveals some interesting features regarding regulation of the gene encoding bS21-2, *rpsU2*. Specifically, in cells lacking bS21-2, there is a substantial increase in transcript abundance corresponding to the *rpsU2* operon in comparison to wild-type cells. This increase in *rpsU2* operon transcript abundance can be complemented by ectopic expression of all three bS21 homologs. Given that in the absence of bS21-2, the *rpsU2* transcript abundance increases so drastically, it follows that bS21-2 is capable of repressing, or autogenously regulating, its own operon. Unlike the zinc mediated r-protein regulation, this autogenous regulation mechanism appears to be more similar to other ribosomal proteins.

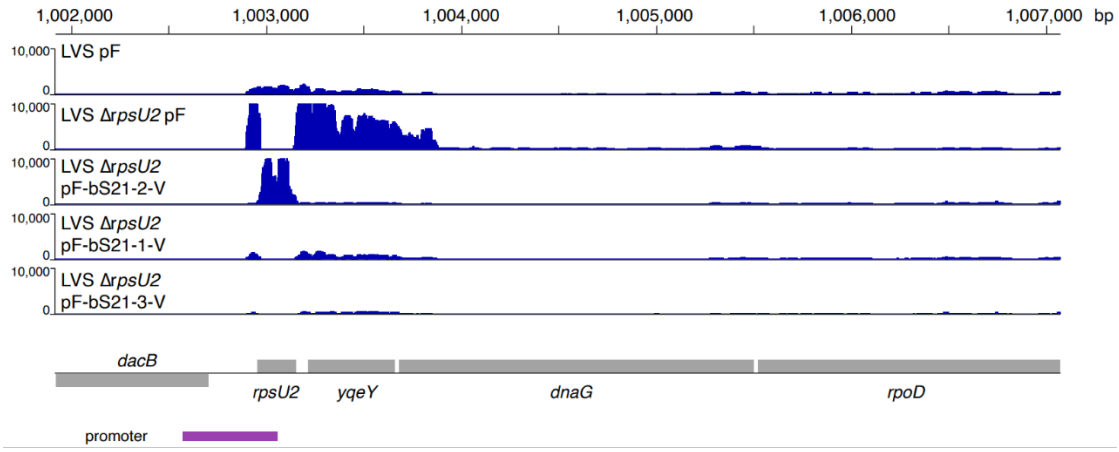

Figure 4. RNA Seq data suggests that bS21-2, bS21-1, and bS21-3 negatively regulate the *rpsU2* operon. Normalized transcript abundance reads from RNA-Seq experiments mapped to the *rpsU2* operon. Data from cells with bS21-2 (LVS pF), cells lacking bS21-2 (LVS Δ*rpsU2* pF), and cells lacking bS21-2 but ectopically expressing either bS21-2, bS21-1, or bS21-3 (LVS Δ*rpsU2* pF-bS21-2-V, pF-bS21-1-V, pF-bS21-3-V, respectively) from a heterologous strong promoter.

bS21-1 and bS21-3 in *F. tularensis*

rpsU1 and rpsU3 operon structure

Less is known about the roles of bS21-1 and bS21-3 in *F. tularensis* and limited information can be gleaned from the genomic context of their genes. The bS21-3-encoding gene, *rpsU3,* is not found within an operon with other annotated genes. The gene encoding bS21-1 is encoded downstream of *cspC*, a cold shock family protein (Trautmann and Ramsey 2022).

rpsU1-like operon in other species and F. tularensis

The location of *rpsU1* adjacent to a cold shock protein is similar to the genetic organization of one of two *rpsU* homologs in *Sinorhizobium meliloti.* Specifically, the *S. meliloti rpsU* homolog is encoded in an operon downstream of two genes, *cspA* and ORF2 (now annotated as SM_RS10560) in *S. meliloti*. ORF2 encodes a novel polypeptide sequence which the authors determined to

respond to cold shock although it does not have similarity to other Csp family proteins. The authors determined that *cspA* could be transcribed as a single mRNA transcript, or a polycistronic mRNA corresponding to the full operon. These transcripts both responded to cold shock, with a larger increase of the polycistronic transcript including the *rpsU* mRNA (105-fold increase). In order to assess the regulation of the *cspA* operon, the authors created fusions of either the promoter or the promoter and most of the 5′ UTR of the *cspA* operon fused to the *luxAB* reporter gene, integrated into the chromosome. Through exposure to cold shock and subsequent total RNA Northern Blots, it was determined that both the promoter and 5′ UTR lead to regulation of the *cspA* operon during cold shock, with more effect due to the presence of the 5′ UTR. Interestingly, the authors also noted that at 30°C, the 5′ UTR decreased transcript abundance, which they suggest may be due to degradation. The authors speculated that bS21 may increase the affinity of mRNAs with ribosomal machinery, which is decreased during cold stress. Contrary to their hypothesis, the authors did not observe a cold-shock susceptible phenotype in *S. meliloti* cells lacking *rpsU*, though they did note that another *rpsU* homolog was present in the genome and that it was possible that this homolog compensated for the deleted *rpsU* (O'Connell and Thomashow 2000).

In *F. tularensis, rpsU1* does not share an operon with *cspA,* but rather *cspC* (Trautmann and Ramsey 2022). In *E. coli* the CspC protein has been found to act as a transcription antiterminator, and may down-regulate heat shock proteins (Bae et al. 2000; Shenhar et al. 2009). Additionally the production of

CspC is not induced by cold-shock (Czapski and Trun 2014). While *rpsU* genes in both *S. meliloti* and *F. tularensis* share an operon with cold shock family proteins, it is currently unclear how similar their regulation and function may be.

The roles of bS21-1 and bS21-3 in F. tularensis

In *F. tularensis*, bS21-1 and bS21-3 are the least abundant homologs (Trautmann and Ramsey 2022). This was determined through mass spectrometry analysis of purified 70S ribosomes. bS21-1 and bS21-3 could not be unambiguously identified due to their high sequence identity, and peptides corresponding to these proteins were only identified in one sample (Figure 5). Additionally, the spectral counts, on account of them being generated from purified ribosomes, shows that bS21-1 and/or bS21-3 can be found in ribosomes. This gives rise to evidence that *F. tularensis* possesses heterogenous ribosomes (Trautmann and Ramsey 2022).

Peptides corresponding to	Spectral counts			
	A	B	C	D
bS21-2	18	11	8	9
bS21-1 and/or bS21-3	3	ND	ND	ND

Figure 5. bS21-1 and bS21-3 are the least abundant homologs in *F. tularensis.* Spectral counts suggesting the number of peptide particles corresponding to either bS21-1/bS21-3 or bS21-2 incorporated into ribosomes of wild-type cells. bS21-1 and bS21-3 could not be further resolved due to peptide similarity. A-D refer to individual ribosomal purifications. ND refers to none detected, indicating that bS21-1/bS21-3 were only detected in sample A (Trautmann and Ramsey 2022).

There is limited information with respect to how bS21-1 and bS21-3 influence translation. Specifically, it was found that ectopic expression of either bS21-1 or bS21-3 could, to some level, restore abundance of T6SS proteins in cells lacking bS21-2 (Trautmann and Ramsey 2022). Particularly, ectopic

expression of bS21-3 in cells lacking bS21-2 was found to restore virulence protein levels to wild-type or higher levels. On the other hand, ectopic expression of bS21-1 in cells lacking bS21-2 only led to partial restoration of wild-type virulence protein levels. However, while cells lacking bS21-2 have reduced fitness within macrophage, intramacrophage growth was only rescued by ectopic expression of bS21-2, not bS21-1 or bS21-3 (Trautmann and Ramsey 2022). This indicates that bS21-2, but not bS21-1 or bS21-3, is specifically required for survival inside macrophage (Trautmann and Ramsey 2022).

While bS21-1 and bS21-3 can independently incorporate into ribosomes, leading to ribosomal heterogeneity, the factors controlling production of these two homologs is still unclear. And although prior data revealed that bS21-2 is autogenously regulated, how bS21-2 exerts its effects on its own production is also unknown. These topics will be explored in the following chapters.

SUMMARY AND OBJECTIVES

Ribosomes may be heterogenous due to a number of factors, including incorporation of distinct ribosomal protein homologs. In our organism of interest, *F. tularensis*, there are three homologs of the ribosomal protein bS21. Previous work in the lab indicated that bS21-2 is the most abundant of the three homologs, but that bS21-1 and bS21-3 can also be assembled into ribosomes. When present in ribosomes, bS21-2 affects the production of certain virulence proteins. How the production of specific bS21 homologs is regulated and how the three homologs are kept in balance with other ribosomal components is not well understood.

In the following chapters, I show that the autogenous regulation of bS21-2 is, at least in part, due to its leader sequence. Additionally, I show that two stem-loops in the leader sequence are not important for regulation by bS21-2, but that bS21-2 may de-stabilize its own transcript. Uniquely, bS21-1 and bS21-3 are not autogenously regulated. In the third chapter, I describe a number of environmental conditions and their impacts on regulation of bS21-1 and bS21-3 at either the transcriptional or translational level.

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CHAPTER 2

Manuscript 1

Control of bS21 homolog production in *Francisella tularensis*

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Abstract

Francisella tularensis is a highly infectious human pathogen that must replicate inside macrophage to cause disease. *F. tularensis* ribosomes can incorporate one of three different homologs for the small ribosomal subunit protein bS21. One of these homologs in particular positively controls key virulence genes and intramacrophage replication. Although ribosomal bS21 content influences *F. tularensis* virulence, the factors that control bS21 homolog production are not well understood. Here, we reveal that all three bS21 homologs influence transcript abundance of the homolog important for virulence, bS21-2. In contrast, the other bS21 homologs (bS21-1 and bS21-3) do not affect their own production. We additionally determined that while a particular 61 nucleotide section of the bS21-2 leader sequence is sufficient for bS21-2-mediated repression of mRNA abundance, translation of the bS21-2 transcript is controlled by other factors. Finally, bS21-2 may exert its effects on bS21-2 transcript by altering its stability. Together, our findings suggest that *F. tularensis* integrates multiple signals into a regulatory network to control the appropriate production of each bS21 homolog, and particularly the homolog important for virulence, bS21-2. This regulatory network in turn may control ribosomal heterogeneity and virulence gene expression.

Introduction

Among the cellular processes, translation is the most energetically costly (Hu et al., 2020). Part of this cost is the assembly of the large molecular machine

that catalyzes protein synthesis, the ribosome. Accordingly, cells have complex regulatory networks to balance the production of rRNA and ribosomal proteins (r-proteins). For example, operons encoding multiple r-proteins are often regulated by an r-protein that is among the first to bind rRNA in assembly (primary binding r-proteins), as these r-proteins recognize and bind specific mRNA features that are structurally analogous to their binding sites on rRNA (Nomura et al., 1984).

It is common for bacteria to encode multiple homologs for at least one ribosomal protein, which can lead to heterogeneity in ribosome composition (Yutin et al., 2012). This also raises the question of how production of different r-protein homologs is coordinated and balanced. Many highly divergent r-protein paralogs either do or do not coordinate zinc (Cheng-Guang and Gualerzi, 2021; Yutin et al., 2012). When in zinc-replete conditions, organisms with these paralogs incorporate the zinc-coordinating r-protein paralog into ribosomes. But in environments with less zinc, cells use zinc-responsive transcription factors to allow production of r-protein paralogs that do not coordinate zinc (alternative paralogs). Subsequently, the alternative paralogs are incorporated into ribosomes. This mechanism of paralog switching, dependent on the concentration of a divalent cation and the de-repression of a transcription factor, is relatively straightforward and is thought to allow dynamic control of intracellular zinc concentrations (Cheng-Guang and Gualerzi, 2021; Shin and Helmann, 2016).

The highly infectious intracellular human pathogen *Francisella tularensis* encodes three distinct homologs of the small ribosomal subunit protein bS21 and we have demonstrated that *F. tularensis* ribosomes can be heterogenous with respect to bS21 content (Trautmann and Ramsey, 2022). Additionally, ribosomes with bS21-2 are uniquely important for both production of a critical virulence factor, the type VI secretion system, and intramacrophage survival, which is essential for *F. tularensis* to cause disease (Trautmann and Ramsey, 2022). How *F. tularensis* cells balance production of the three bS21 homologs is not clear. bS21 is not a primary binding r-protein (it is among the last to be assembled into the ribosome) and none of the *F. tularensis* bS21 homologs are encoded in operons with other primary binding r-proteins (Mizushima and Nomura, 1970; Trautmann and Ramsey, 2022). None of the *F. tularensis* bS21 homologs are predicted to coordinate zinc or other divalent cations. However, there is evidence that, in *F. tularensis*, bS21-2 is autogenously regulated; in cells without bS21-2, there is a large increase in mRNA corresponding to the gene immediately downstream of the bS21-2 gene, *yqeY*, which can be complemented by ectopic expression of bS21-2 (Trautmann and Ramsey, 2022).

There is limited information regarding control of bS21 in other organisms. *E. coli* encodes only a single bS21 homolog which is not autogenously regulated (Nomura et al., 1984; Takata, 1978). In the Bacteroidia species *Flavobacterium johnsoniae*, the single bS21 homolog is autogenously controlled, but through a mechanism unlikely to be present in divergent species such as *F. tularensis*.

Specifically, the C-terminal region of *F. johnsoniae* bS21 is conserved among Bacteroidia species and contributes to the sequestration of the anti-Shine-Dalgarno (ASD) (Jha et al., 2020; McNutt et al., 2023). This sequestration precludes ribosomes from being responsive to Shine-Dalgarno (SD) sequences, but the transcript encoding bS21 is essentially the only *F. johnsoniae* mRNA with a strong SD. Thus, when bS21 is depleted and the ASD becomes accessible, translation of the bS21-encoding transcript increases. However, this mechanism for autogenous regulation is likely limited to the bS21 homologs in Bacteroidia with conserved sequence and structure in their C-terminal region.

In *F. tularensis*, we measured RNA abundance in cells with and without bS21-2 and found that the presence of any of the three *F. tularensis* bS21 homologs reduces steady-state levels of the bS21-2-encoding transcript, *rpsU2*. This autogenous regulation appears to be unique to bS21-2, as we did not find that bS21-1 or bS21-3 similarly control their own production. Using reporter assays, we found that despite the increase in *rpsU2* transcript abundance in cells without bS21-2, there is no concordant increase in protein abundance, suggesting that other regulatory mechanisms control *rpsU2* translation. Additional reporter assays demonstrated that while the 5´ UTR of *rpsU2* is sufficient for control of transcript abundance by bS21-2, the first 38 nucleotides of the *rpsU2* transcript, predicted to encode two stem-loops, are dispensable for regulation. Finally, bS21-2 may exert its effects on *rpsU2* abundance by altering mRNA stability. Our results suggest that control of the bS21 homologs in *F.*

tularensis, and particularly of bS21-2, is complex and regulated by multiple factors.

Results

bS21 homologs control abundance of the bS21-2-encoding mRNA

Previous studies examined the effects of bS21-2 deletion on mRNA abundance using RNA-Seq (Trautmann and Ramsey, 2022). Notably, the gene with the largest change in transcript abundance, *yqeY*, is encoded in the same operon and immediately downstream of the bS21-2-encoding gene, *rpsU2*. Loss of bS21-2 leads to a 6-fold increase in *yqeY* transcript, which can be complemented by ectopic expression of bS21-2. Further analysis reveals that the region corresponding to the 5´ UTR of *rpsU2* is increased 9-fold in the absence of bS21-2 (**Fig 1**). These results suggest that bS21-2 autogenously controls its own production, functioning as a negative regulator of the *rpsU2* operon transcript.

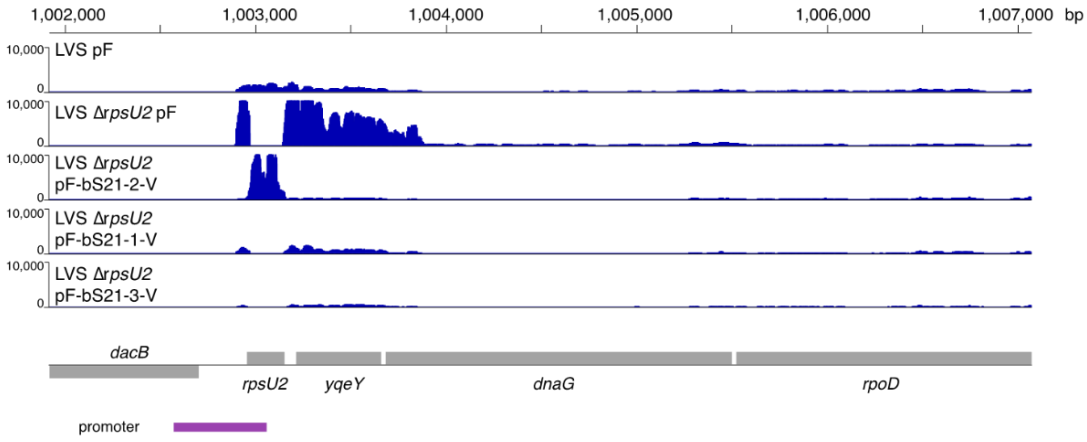


Figure 1. All three *F. tularensis* bS21 homologs negatively regulate the *rpsU2* operon. Normalized transcript abundance reads from RNA-Seq experiments in the area surrounding the *rpsU2* operon. Data from cells with bS21-2 (LVS pF), cells lacking bS21-2 (LVS Δ*rpsU2* pF), and cells lacking bS21-2 but ectopically expressing either bS21-2, bS21-1, or bS21-3 (LVS

Δ*rpsU2* pF-bS21-2-V, pF-bS21-1-V, pF-bS21-3-V, respectively). Y-axis is truncated at 10,000 for clarity. Grey rectangles represent genes and those above the line indicate genes encoded on the positive strand; those below the black line represent those encoded on the negative strand. The purple box indicates an experimentally-determined promoter region (Ramsey et al., 2015).

In addition to bS21-2 controlling its own transcript, additional unpublished RNA-Seq data reveal that the other bS21 homologs can also repress expression of the *rpsU2* operon. In particular, ectopic expression of either bS21-1 or bS21-3 in cells lacking bS21-2 lead to reductions in the *rpsU2* transcript abundance to levels similar to that found in wild-type cells (**Fig 1**). Thus, all three bS21 homologs function similarly with respect to repressing abundance of mRNA corresponding to the *rpsU2* operon.

bS21-1 and bS21-3 do not control their own production

While loss of bS21-2 did not affect the transcript abundance of the genes encoding bS21-1 or bS21-3 (*rpsU1* and *rpsU3*, respectively), (Trautmann and Ramsey, 2022) finding that bS21-2 autoregulates its production raises the possibility that other bS21 homologs in *F. tularensis* may also be autoregulated. To test this possibility, qRT-PCR was used to assess the abundance of the transcripts encoding these proteins in cells with and without the r-protein of interest. Specifically, we compared transcript abundance of the 5´ UTR of *rpsU1* in cells with and without bS21-1 (**Fig 2A**) and the 5´ UTR of *rpsU3* in cells with and without bS21-3 (**Fig 2B**). In contrast to our findings regarding the control of bS21-2, loss of the other bS21 homologs does not appear to impact the abundance of their own transcripts.

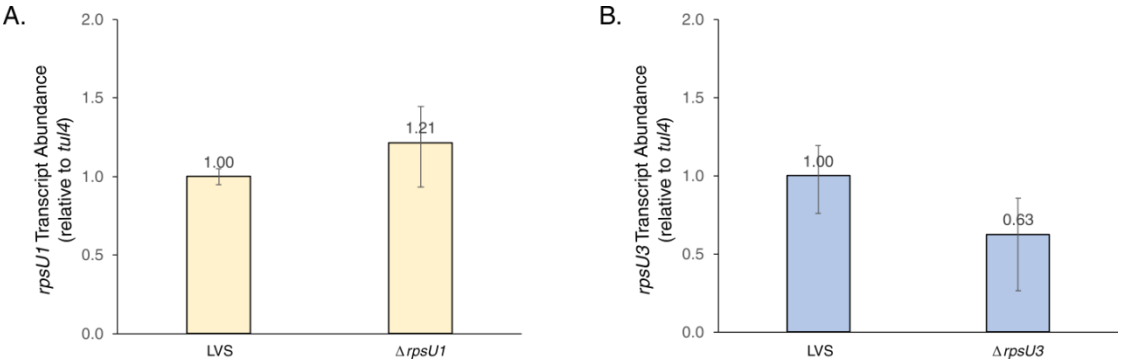


Figure 2. bS21-1 and bS21-3 do not regulate their own transcript abundance. (A) Transcript abundance of the *rpsU1* 5´ UTR in either wild-type cells or cells lacking *rpsU1*, relative to *tul4* (a control gene). (B) Transcript abundance of the *rpsU3* 5´ UTR in either wild-type cells or cells lacking *rpsU3*, relative to *tul4* (a control gene). (A and B) Differences did not reach statistical significance by *t* test.

Transcription and translation of the *rpsU2* operon are differentially regulated

We generated translational reporter fusions to further examine autoregulation of bS21-2. The transcription start site for *rpsU2* was previously identified, allowing us to use a defined 5´ UTR (Ramsey et al., 2015). The first translational reporters included the promoter, 5´ UTR, and the first six codons of either *rpsU2* or a control gene, *tul4*, fused to *lacZ* (**Fig 3A**). These reporters were incorporated in single copy into the chromosome at the Tn7 site in cells with or without bS21-2. We measured transcript abundance of the *lacZ* gene using qRT-PCR and translation using ß-galactosidase assays. We found that the *rpsU2* translational reporter recapitulates control of *rpsU2* mRNA abundance by bS21-2; in cells lacking bS21-2, *lacZ* transcript increases by ~9-fold (**Fig 3B**). In contrast, the amount of *lacZ* transcript is essentially the same in cells with the *tul4* reporter, regardless of bS21-2 presence (**Fig 3B**). However,

translation of the *lacZ* mRNA with the *rpsU2* leader sequence is not affected by bS21-2 in the same way. Despite the ~9-fold increase in *lacZ* transcript, there is only ~30% more ß-galactosidase activity in cells lacking bS21-2 compared to cells with bS21-2 (**Fig 3C**). Thus, it seems that changes in bS21-2-controlled transcript abundance are not reflected in translation.

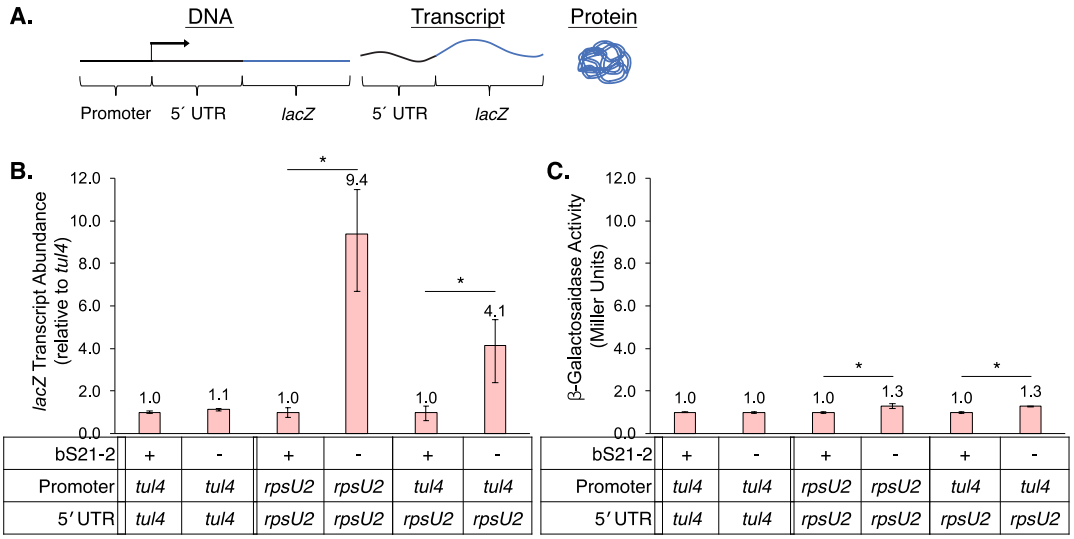


Figure 3. The *rpsU2* 5´ UTR allows control transcript abundance yet transcript and protein abundance changes are not correlated. (**A**) Diagram of translational reporters. (**B**) Relative *lacZ* transcript abundance for indicated translational fusions in cells with (+; wild-type) or without (-; Δ*rpsU2*) bS21-2. Quantitative RT-PCR was used to determine the relative transcript normalized to the *tul4* gene. (**C**) Relative β-galactosidase activity for indicated *lacZ* translational fusions in cells with (+; wild-type) or without (−; Δ*rpsU2*) bS21-2. (**B and C**) Error bars represent 1 SD. Lines above bars indicate comparisons, *p < 0.05. Experiments were repeated at least twice in biological triplicate, and data from a representative experiment are shown.

There is evidence that this control is not unique to translation of our reporter construct but is important for translation of the native *rpsU2* transcript. Specifically, while the abundance of the *rpsU2* operon transcript, including *yqeY*, is significantly increased in cells without bS21-2, proteomic analysis revealed no significant change in YqeY protein abundance (changes reported as

significant are 1.5-fold altered with an adjusted $P < 0.05$; (Trautmann and Ramsey, 2022)). These findings suggest that while bS21-2 regulates the abundance of its own transcript, one or more additional factors control translation of the bS21-2 transcript.

The *rpsU2* 5´ UTR is sufficient for transcriptional autoregulation

There are a variety of mechanisms by which production of r-proteins are controlled and many of them depend on mRNA leader sequences of the regulated r-protein. To determine if the 5´ UTR of *rpsU2* is sufficient to permit regulation by bS21-2, we created translational fusions which used a control promoter (*tul4*) driving expression of the 5´ UTR and first six codons of *rpsU2* fused to *lacZ*. As with the other reporters, this reporter was integrated onto the chromosome at the Tn7 site in cells with and without bS21-2. We found that in cells without bS21-2, there was ~4-fold more *lacZ* transcript compared to cells with bS21-2, suggesting that the *rpsU2* 5´ UTR is sufficient to allow at least some control by bS21-2 (**Fig 3B**). Additionally, using this reporter we observed the same disconnect between control of transcript abundance and translation. Despite the ~4-fold increase in *lacZ* transcript abundance, we only found ~30% more ß-galactosidase activity in cells lacking bS21-2 compared to cells with bS21-2 (**Fig 3C**). Thus, the *rpsU2* 5´ UTR is sufficient to lead to repression of mRNA abundance by bS21-2 and regulation of translation by other factor(s).

Stem-loops in the *rpsU2* mRNA do not influence regulation by bS21-2

We sought to investigate what features of the 5´ UTR allow bS21-2 to control the abundance of the *rpsU2* mRNA. The secondary structures formed

by mRNA leader sequences are often subject to regulatory control and the first 38 nucleotides of the *rpsU2* 5´ UTR are predicted to form two stem-loop structures (**Fig 4A**). We considered the possibility that these stem-loops might be key for regulation of *rpsU2* mRNA abundance and generated plasmid-based reporter fusions to test this hypothesis. Specifically, these reporters included either the full-length 5´ UTR (91 nt; "full") or truncated version (61 nt; "Δloops") and the first six codons of *rpsU2* fused in-frame with *gfp*, driven by the control promoter (*tul4*). The plasmid reporters were introduced into cells with (LVS) and without bS21-2 (Δ*rpsU2*) and we measured both *gfp* transcript abundance and GFP fluorescence (**Fig 4B, C**). Cells containing the reporter construct with the full-length *rpsU2* 5´ UTR recapitulated our previous observations regarding regulation of the *rpsU2* mRNA, specifically that transcript abundance increases significantly (>2-fold) in cells without bS21-2, but that there is no similarly meaningful increase in protein abundance. Notably, cells containing the truncated *rpsU2* 5´ UTR reporter construct exhibited the same phenotypes (**Fig 4B, C**). These results indicate that the first 38 nt of the *rpsU2* mRNA, including any predicted stem-loops, are dispensable for regulation by bS21-2.

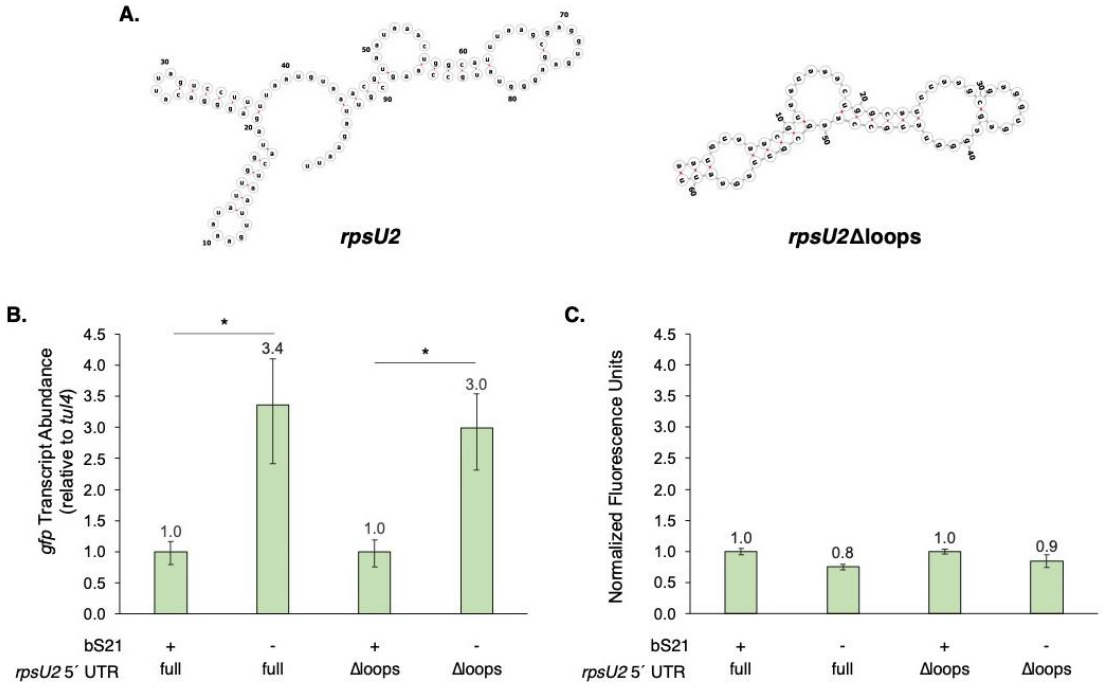
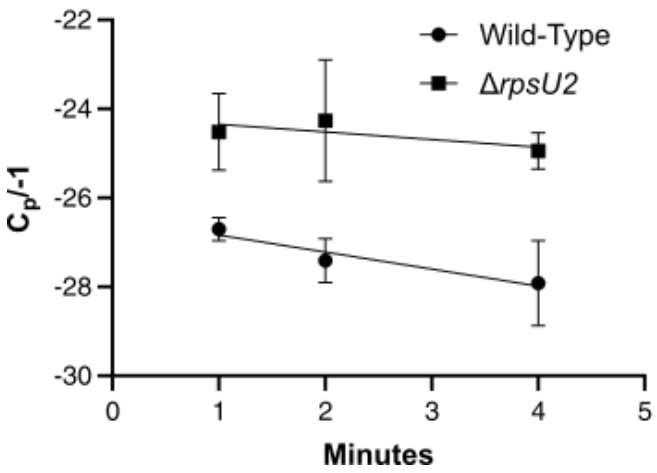


Figure 4. Predicted stem-loops in the *rpsU2* mRNA are dispensable for control by bS21-2. (**A**) Secondary structure predictions of wild-type and modified *rpsU2* 5´ UTRs, generated by MXfold2. (**B**) Relative *gfp* transcript abundance for indicated translational fusions in cells with (+; wild-type) or without (-; Δ*rpsU2*) bS21-2. Quantitative RT-PCR was used to determine the relative transcript normalized to the *tul4* gene. (**C**) Relative fluorescence of cells with indicated *gfp* translational fusions in cells with (+; wild-type) or without (−; Δ*rpsU2*) bS21-2. (**B and C**) Error bars represent 1 SD. Lines above bars indicate comparisons, *p < 0.05. Experiments were repeated at least twice in biological triplicate, and data from a representative experiment are shown.

bS21-2 may influence the stability of its own mRNA

The amount of any given transcript in a cell depends on two factors: the amount of new transcript produced and the rate of its degradation. Since we determined that the *rpsU2* 5´ UTR is sufficient for bS21-2-mediated changes in abundance, we considered that stability, rather than production, may be a key factor in regulating *rpsU2* transcript abundance. We used cells with and without bS21-2 and used a transcription inhibition assay to determine the stability of the

rpsU2 operon transcript. Specifically, we grew cells to mid-log phase, added the RNA polymerase inhibitor rifampicin to halt transcription initiation, and isolated RNA at several timepoints. We subsequently used qRT-PCR to determine the amount of RNA corresponding to the *yqeY* transcript at each timepoint. Using this approach, we have consistently found that the *rpsU2* transcript has a longer half-life in cells lacking bS21-2 (~6 minutes compared to ~2.5 minutes; **Fig 5**). However, variability within these experiments precludes these results from being statistically significant, so we cannot conclusively say that the presence of bS21-2 destabilizes its own transcript.



	Half-Lives (min)
Wild-Type	2.6
Δ*rpsU2*	5.8

Figure 5. The presence of bS21-2 may destabilize the *rpsU2* mRNA. Left: Graph of *rpsU2* operon transcript over time after transcription inhibition. C_p/-1 reflects qRT-PCR crossing point (Cp) divided by -1, and reflects total RNA amount; numbers closer to 0 represent more mRNA. Linear regression analysis was used to fit data (Prism), but slopes are not significantly different ($p = 0.37$). Error bars represent 1 SD. Right: Half-lives calculated from slope of best-fit values. Experiments were repeated at least twice in biological triplicate, and data from a representative experiment are shown.

Discussion

In this study, we investigate what factors allow coordinated production of bS21 homologs in *F. tularensis*. In particular, we determined that the presence of any of the three bS21 homologs can repress abundance of the transcript encoding bS21-2, *rpsU2*, but neither bS21-1 nor bS21-3 influence the abundance of their own transcripts. However, our work reveals that translation of the bS21-2 protein is further controlled by unknown factor(s), as large increases in *rpsU2* transcript abundance in cells without bS21-2 do not lead to similar increases in protein abundance. We further identify that while the 5´ UTR of the *rpsU2* transcript is sufficient to lead to regulation by bS21-2, two predicted stem-loops in the leader sequence are not required. Finally, our work suggests that bS21-2 may exert its effects on *rpsU2* transcript abundance by altering mRNA stability. Together, these results reveal that coordinated production of bS21 homologs, and particularly bS21-2, in *F. tularensis* is complex and controlled at multiple steps.

The observation that the presence of any of the three bS21 homologs can reduce *rpsU2* transcript abundance is consistent with a model in which r-proteins are regulators in a negative feedback loop. In particular, the abundance of an r-protein would be inversely correlated to the abundance of its mRNA (i.e., as the r-protein abundance decreases, its transcript increases). In this case, all three bS21-2 homologs can repress the transcript encoding bS21-2. These bS21 homologs are between 48-72% identical (Trautmann and Ramsey, 2022), and, given their similar influence on *rpsU2* mRNA, it raises the possibility that

the conserved residues in bS21 homologs are key for regulation of *rpsU2*, either directly or indirectly.

The logical extension of this negative feedback model is ultimately a balance in r-protein abundance, with lower amounts of an r-protein triggering production of more r-protein and higher amounts inhibiting excess production. Yet, despite a 3-fold increase in bS21-2-encoding transcript, we observed only about a 30% increase in bS21-2 protein using reporter systems and when examining control of genes in the native *rpsU2* operon. Because the presence or absence of bS21-2 does not substantially alter the abundance of proteins encoded by the *rpsU2* operon, we hypothesize that another factor is limiting translation initiation, even in the presence of abundant *rpsU2* mRNA. This factor (or factors) remains to be identified.

While the 5´ UTR of the *rpsU2* transcript is sufficient for regulation by bS21-2, the specific elements that permit regulation have yet to be identified. In examining the contribution of two predicted stem-loops in the leader sequence, we narrowed down the region sufficient to lead to regulation to 61 nucleotides. Additional work will be required to identify what sequence elements contribute to this regulation, as well as how bS21-2 exerts its regulatory effects.

Materials and Methods

Bacterial strains and growth conditions

F. tularensis subsp. *holarctica* LVS cells were grown in Mueller Hinton Broth supplemented with 0.025% iron pyrophosphate, 0.1% glucose, and 2% Isovitalex at 37°C shaking aerobically, or on cystine heart agar plates containing 1% hemoglobin (CHA-H) at 37°C. *E. coli* XL1-Blue and PIR1 cells were grown in LB media or on LB plates. The selective antibiotics kanamycin (kan), hygromycin (hyg), or nourseothricin (nat) was used to maintain plasmids in *F. tularensis* LVS at 5 µg/mL or in *E. coli* at 50 µg/mL.

Integration of reporter constructs into the Tn7 site of F. tularensis

Reporter constructs were integrated into the Tn7 site of *F. tularensis* LVS as previously described (LoVullo et al., 2009). Briefly, LVS cells were made electrocompetent by washing with 10% sucrose. The Tn7 helper plasmid pMP720 was electroporated into electrocompetent cells with or without bS21-2, and plasmid-containing cells were selected on hygromycin B-containing media. Cells harboring the helper plasmid were electroporated with the appropriate mini-Tn7 plasmid and selected for on CHA-H plates with kanamycin. PCR was used to screen kanamycin-resistant colonies for plasmid integration at the attTn7 site and candidates were confirmed by amplification and sequencing of gDNA outside of the attTn7 site.

Plasmids

To generate plasmids in which the *rpsU2* 5´ UTR is upstream of the *gfp* coding sequence, pKR123 (unpublished) was digested with KpnI/NotI, resulting

in a fragment containing the promoter of *tul4* fused to the *rpsU2* 5´ UTR. Another plasmid, pKR183, containing the sfGFP gene codon-optimized for expression in *F. tularensis* LVS (Trautmann et al., 2023), was digested with KpnI/BamHI. Further, pKR123 (unpublished), containing the mini-Tn7 plasmid backbone was digested with NotI/BamHI, and all three of these fragments were ligated together forming the in-frame GFP reporter plasmid, pKR184 Tn7 P*tul4-rpsU2* 5´ UTR-GFP.

To create a plasmid in which the Δloops 5´ UTR is upstream of the *gfp* coding sequence, primers were used to amplify from pKR184, deleting the stem loop structures in the first 38 nt of the *rpsU2* 5´ UTR. The first primer contained a PacI site with the last 22 nt of the promoter of *tul4* on the 5´-end fused to the 32 nt, directly after the first 38 nt of the *rpsU2* 5´ UTR which are not encoded on the primer. The reverse primer located 152 bp upstream of the 3´ end of the *gfp* gene contained the MfeI cut site. The resultant fragment was ligated with PacI/MfeI digested pKR184, effectively deleting the stem loop structures and resulting in pKR191 Tn7 P*tul4-rpsU2* Δloops 5´ UTR-GFP.

Strains

The derivatives of *F. tularensis* subsp. *holarctica* LVS used in this study are found in **Table 1**.

Table 1. *F. tularensis* LVS strains used in this study

Strain	Genotype	Integrated Plasmid
KRLVS111	LVS *ΔrpsU2* Tn7::P*tul4 tul4* 5'UTR-*lacZ aphA*	pKR89 Tn7::P*tul4-tul4* 5'UTR-6aa- *lacZ*_R6Kg
KRLVS112	LVS Tn7::P*tul4 tul4* 5'UTR-*lacZ aphA*	pKR89 Tn7::P*tul4-tul4* 5'UTR-6aa- *lacZ*_R6Kg

KRLVS148	LVS Δ*rpsU2* Tn7::P*rpsU2 rpsU2* 5'UTR-*lacZ aphA*	pKR121 Tn7::P*rpsU2 rpsU2* 5'UTR-*lacZ*_aphA
KRLVS149	LVS Tn7::P*rpsU2 rpsU2* 5'UTR-*lacZ aphA*	pKR121 Tn7::P*rpsU2 rpsU2* 5'UTR-*lacZ*_aphA
KRLVS150	LVS Δ*rpsU2* Tn7::P*tul4 rpsU2* 5'UTR_*lacZ aphA*	pKR123 Tn7::P*tul4 rpsU2* 5'UTR-*lacZ*_aphA
KRLVS151	LVS Tn7::P*tul4 rpsU2* 5'UTR_*lacZ aphA*	pKR123 Tn7::P*tul4 rpsU2* 5'UTR-*lacZ*_aphA
KRLVS279	LVS Tn7::P*tul4 rpsU2* 5'UTR-*gfp*	pKR184 Tn7::P*tul4 rpsU2* 5'UTR -*gfp*
KRLVS280	LVS Δ*rpsU2* Tn7::P*tul4 rpsU2* 5'UTR-*gfp*	pKR184 Tn7::P*tul4 rpsU2* 5'UTR -*gfp*
KRLVS281	LVS Tn7::P*tul4 Δloops rpsU2* 5'UTR-gfp	pKR191 Tn7::P*tul4 Δloops rpsU2* 5'UTR -*gfp*
KRLVS282	LVS Δ*rpsU2* Tn7::P*tul4 Δloops rpsU2* 5'UTR-gfp	pKR191 Tn7::P*tul4 Δloops rpsU2* 5'UTR -*gfp*

mRNA stability assay

The stability of mRNAs was assessed essentially as described (Nguyen et al., 2020). Briefly, strains of interest were grown to mid-log phase (OD_{600} = 0.3 – 0.4) in triplicate, and rifampicin was added to a final concentration of 50 ug/mL. After 0, 1, 2, 4, and 8 minutes, 7 mL cultures were snap frozen using liquid nitrogen and stored at -80°C until purification.

RNA isolation and quantitative real-time PCR

F. tularensis LVS cells with and without bS21-2 (KRLVS148 and KRLVS149) were grown to mid-log (OD_{600} = 0.3 – 0.4) in triplicate. Total nucleic acids were isolated using the Zymo Research Direct-zol RNA Kit following manufacturer's protocols. Nucleic acids were treated with RQ1 DNase (Promega) and RNA was re-purified using the Zymo Research Direct-zol RNA Kit. cDNA was synthesized and quantitative real-time PCR was performed as

previously described (Trautmann and Ramsey, 2022). Experiments were performed at least twice in biological triplicate.

β-Galactosidase assay

Indicated derivatives of *F. tularensis* LVS cells were grown to mid-log (OD_{600} = 0.3 – 0.4) in triplicate and ß-galactosidase activity was measured as previously described (Charity et al., 2009). Reactions were stopped after 120 minutes if no significant yellow color developed. Experiments were performed at least twice in biological triplicate.

GFP assay

Indicated derivatives of *F. tularensis* LVS cells were grown to mid-log (OD_{600} = 0.3 – 0.4) and 1 - 4mL were pelleted and resuspended in PBS. Optical density (OD_{600}) and fluorescence (excitation 495 nm, emission of 535 nm) were measured in technical triplicate using a SpectraMax® iD3 Multi-Mode Microplate Reader. Fluorescence readings were normalized to OD_{600} and the fluorescence from LVS cells (containing an empty vector but no GFP reporter) was subtracted. Experiments were conducted at least twice in biological triplicate.

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CHAPTER 3

Manuscript 2

Identification of conditions that lead to changes in bS21-1 and bS21-3 abundance in *Francisella tularensis*

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Abstract

Francisella tularensis is a highly infectious, intracellular human pathogen that can cause fatal disease. We have recently found that ribosomes can contain one of the three homologs for the small ribosomal subunit protein bS21. The most abundant homolog, bS21-2, positively controls key virulence genes and intramacrophage replication. However, little is known about the less abundant homologs, bS21-1 and bS21-3, including what conditions might lead to their increased abundance. This study aimed to address this gap in knowledge, by evaluating the production of bS21-1 and bS21-3 after altering environmental factors known affect regulation of other proteins in *F. tularensis*. For most of these conditions, no significant change was observed in production of either bS21-1 or bS21-3. However, bS21-1 was found to be upregulated by exposure of cells to acid stress or growth in different complex media. Similarly, growth in different complex media resulted in increased bS21-3. While this study serves as an initial survey of factors that could impact bS21-1 and bS21-3 production, much remains to be learned about how these proteins are regulated and how they function in *F. tularensis*.

Introduction

Francisella tularensis, the causative agent of tularemia, is a Gram-negative, facultative intracellular bacterium with the potential to cause fatal human disease (Keim et al., 2007). Characteristic of an intracellular pathogen, *F. tularensis* has a reduced genome size. Previous work established that it contains three copies of the gene encoding the small subunit ribosomal protein

bS21, but the function of these three different homologs is still being investigated (Trautmann & Ramsey, 2022).

The role of bS21 in the ribosome is linked to translation initiation across species (Chang & Craven, 1977; Van Duin & Robert, 1981). In *F. tularensis*, the genome encodes three homologs of bS21. The three homologs are bS21-1, bS21-2, and bS21-3, encoded by *rpsU1*, *rpsU2*, and *rpsU3* respectively. Work studying the second homolog, bS21-2, has implicated it in the regulation of several virulence proteins and it has been demonstrated to be autogenously regulated (H. Trautmann & Ramsey, 2022; H. S. Trautmann et al., 2023). However, less is known about the other homologs, bS21-1 and bS21-3, in regard to both their function and their regulation. This lack of information is possibly related to the relatively low abundance of these two proteins in *F. tularensis* LVS grown under standard *in vitro* conditions; they are the bS21 homologs that are least abundant in ribosomes (H. Trautmann & Ramsey, 2022). Knowing that bS21-2 influences the *F. tularensis* proteome, we hypothesize that bS21-1 and bS21-3 may regulate the proteome when there are present and incorporated into ribosomes, in some yet-to-be determined conditions. In an effort to both understand what role they may have in the cell as well as to increase their abundance for easier study, we decided to screen a number of conditions looking for differences in bS21-1 and bS21-3 abundance.

While the environmental reservoir of *F. tularensis* is not known, it is often associated with water sources (Berrada & Telford III, 2011; Broman et al., 2010; Larson et al., 1955). When *F. tularensis* enters a host environment, human or

mammal, it must survive within and escape from the phagosome, prior to cytosolic replication (Celli & Zahrt, 2013). Thus, it must be able to withstand environmental pressures and conditions prior to entering the host environment, withstanding host defenses and inconsistent nutrient states. This necessitates the ability of *F. tularensis* to have both efficient and effective regulatory mechanisms to survive in a variety of environments.

During the transition between environmental sources and a mammalian host, there is a significant change in temperature. Approximately 11% of *F. tularensis* genes are differently regulated during the transition from 26°C (representative of an environmental temperature) to 37°C. Several of these genes are virulence genes, suggesting that temperature-associated regulation is important for initiation of host colonization and virulence (Horzempa et al., 2008).

F. tularensis has been found to survive in brackish waters. The effects of sodium chloride concentration on gene regulation in *F. tularensis* has not been extensively studied. However *F. tularensis* Type A can survive longer in filter-sterilized brackish water compared to filter sterilized freshwater (Berrada & Telford III, 2011). Although it is not clear what leads to increased survival, at least one *F. tularensis* regulatory protein can mediate the response to salt stress. Specifically, the regulator Hfq, an RNA binding protein, is important for both stress response and virulence in mice. *F. tularensis* cells lacking Hfq are more sensitive to a high NaCl concentration (2%) (Meibom et al., 2009).

Changing pH can occur in a variety of environments, but most significantly affects *F. tularensis* after they are engulfed in phagosomes, as the phagosomes mature and acidify. With regard to gene regulation in response to pH, some is known, again, about specific proteins but not necessarily broadly. Specifically, the virulence gene *iglA*'s mRNA transcript and the general regulator, ppGpp, were both found to have decreased levels in acidified environments (pH 5.5), and increased abundance in alkalized environments (pH 8.5) (Faron et al., 2013). Meanwhile, a cytoplasmic membrane protein, RipA, was found to be necessary for growth at neutral pH and to have similar expression levels to IglA during host cell infection. It was also found to be reduced in its expression in lower pHs (Fuller et al., 2009).

F. tularensis is exposed to hydrogen peroxide within phagosomes inside host cells. A number of proteins of *F. tularensis* are differently regulated upon exposure to hydrogen peroxide. Global studies of *F. tularensis* found several proteins which were differently regulated after exposure to hydrogen peroxide, many of which are members of stress response protein families (Ericsson et al., 1994; Lenco et al., 2005).

Within the environment it is possible that *F. tularensis* encounters some UV exposure. It may respond to this stress similarly to other DNA damaging agents, however to the best of our knowledge, there are no current reports on the *F. tularensis* response UV-induced stress.

When inside the host, *F. tularensis* is exposed to an iron-starvation environment. A number of studies have evaluated the effects of iron starvation

on gene expression in *F. tularensis*. A global analysis of gene expression found that at least 80 genes were either up- or down-regulated at least two-fold in iron poor environments. Some of these genes contain a Fur box, a 19 nucleotide consensus DNA sequence, upstream of the gene affecting expression. The Fur box is bound by the transcription factor Fur when it coordinates ferrous iron, repressing gene expression in iron-replete conditions. Among the genes regulated by Fur and increased in iron-depleted environments are those encoding a siderophore, a molecule important for iron acquisition. Additionally, some virulence genes were found to be up-regulated (Deng et al., 2006; Lenčo et al., 2007; Sullivan et al., 2006).

Similarly to iron, *F. tularensis* is exposed to a magnesium-poor environment within hosts. Two major genes have been identified to be differently regulated in magnesium-deplete environments in *F. tularensis*. Like with iron starvation, these genes appear to play a role in magnesium uptake. However, they have also been identified as important for virulence as deletion of these genes, *fmvA* and *fmvB,* led to attenuation in mice (Wu et al., 2016).

In the host environment, *F. tularensis* can be exposed to spermine, a natural polyamine used by eukaryotes for normal cell maintenance (Igarashi & Kashiwagi, 2019). A study looking at the effects of spermine on *F. tularensis* revealed that the presence of spermine influences gene expression. In particular, insertion sequence (IS) elements and nearby genes within the *F. tularensis* genome appeared to be induced by the presence of spermine (Carlson et al., 2009).

As with any microbe, a number of *in vitro* growth conditions have been explored for laboratory cultivation of *F. tularensis.* One of these *in vitro* growth media is Chamberlain's Defined Medium (CDM). Growth in CDM has been found to generally increase the abundance of *F. tularensis* virulence proteins (Carlson et al., 2007; Meibom & Charbit, 2010).

Another medium commonly used in lab cultivation of *F. tularensis* is cysteine heart agar with hemoglobin (CHAH). To the best of our knowledge, there are no reports comparing gene regulation or response to growth on CHAH of *F. tularensis* as compared to other media types.

Results

bS21 homologs are not responsive to a number of conditions

In order to efficiently test multiple conditions to assess relative expression of bS21-1 and bS21-3, we constructed plasmids encoding fluorescent reporters. In particular, these reporters contained the predicted promoter region for either *rpsU1* or *rpsU3*. Because *rpsU1* is the second gene in an operon, the region predicted to encode the *rpsU1* promoter is upstream of the first gene in the operon, *cspC*. The DNA including putative promoter regions (approximately 250 bp for *rpsU1* and approximately 400 bp for *rpsU3*), up to the start codon of either *cspC* or *rpsU3,* is followed by DNA specifying a triple alanine linker in frame with the green fluorescent protein gene (*gfp*). These constructs were created on a multi-copy plasmid and transformed into wild-type *F. tularensis* LVS cells. Thus, we expect that fluorescence will report the relative abundances of either bS21-1 or bS21-3.

Screening for factors that influence expression of rpsU1

When *F. tularensis* cells containing the *rpsU1* reporter were grown to mid-log phase and then exposed to either heat or cold-shock, there was no significant change in fluorescence, compared to cells grown at the standard temperature, 37°C, in standard growth medium (supplemented Mueller-Hinton Broth, sMHB) (**Fig 1A**). Similarly, there were no changes in fluorescence of cells grown in different salt concentrations (**Fig 1B**), or when exposed to either H_2O_2 or UV-stress (**Fig 1C, D**). The conditions tested on *rpsU1* in CDM also showed no significant change in *rpsU1* reported fluorescence in response to changes in iron, magnesium, or spermine concentrations (**Fig 2A, B, C**).

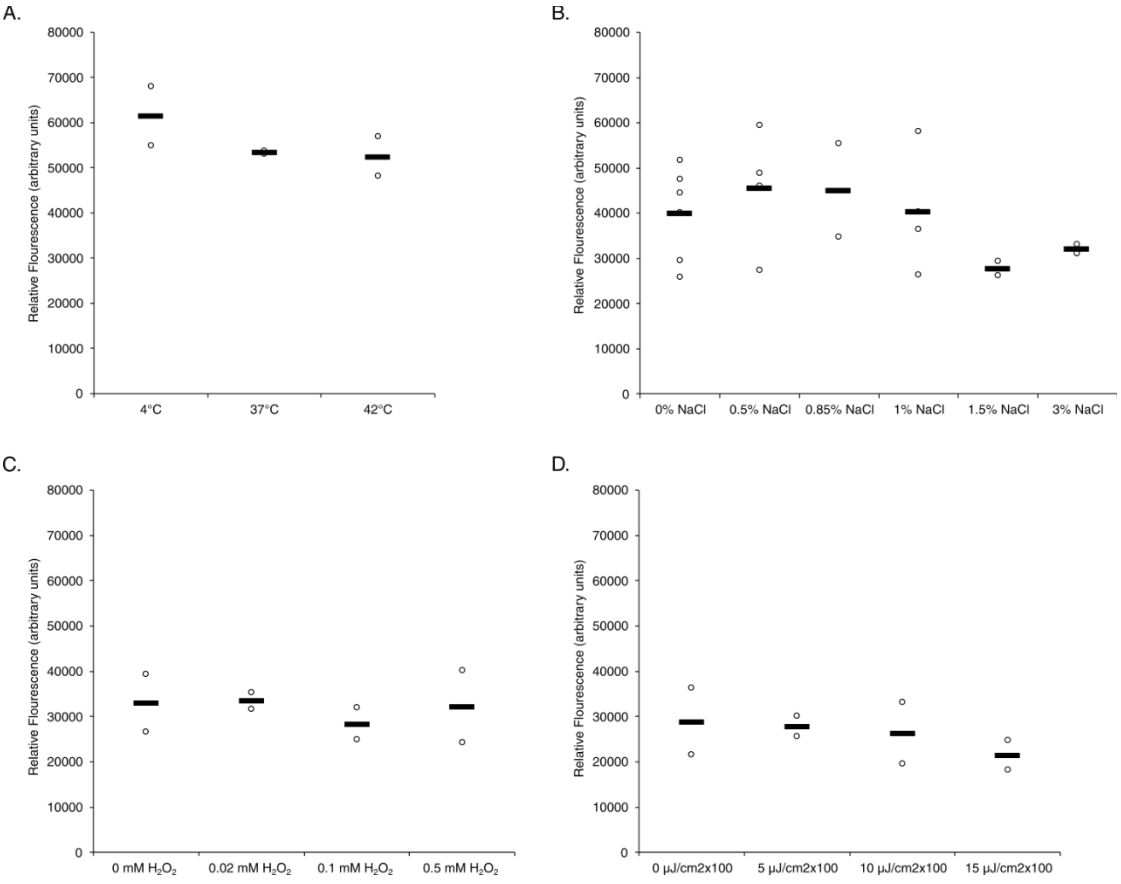

Figure 1. *F. tularensis* cells exposed to different temperatures, salt concentrations, or amounts of oxidative or UV stress do not appear to

have changes in *rpsU1* production. Relative fluorescence values (y-axis) versus environmental conditions (x-axis) of cells containing fluorescent *rpsU1* reporter construct. Each dot represents one replicate, and horizontal bars indicate the average. (**A**) Cells exposed to temperatures of 4°C or 42°C as compared to standard growth temperature, 37°C. Experiment was completed in biological duplicate. (**B**) Cells grown in different salt concentrations. 0.5% NaCl and 1% NaCl were tested twice in biological duplicate, the rest were completed once in biological duplicate. (**C**) Cells grown to log-phase and then exposed to indicated concentrations of hydrogen peroxide (H_2O_2). All were completed once in biological duplicate. (**D**) Cells grown to log-phase and then exposed to indicated doses of UV light exposure. All were completed once in biological duplicate.

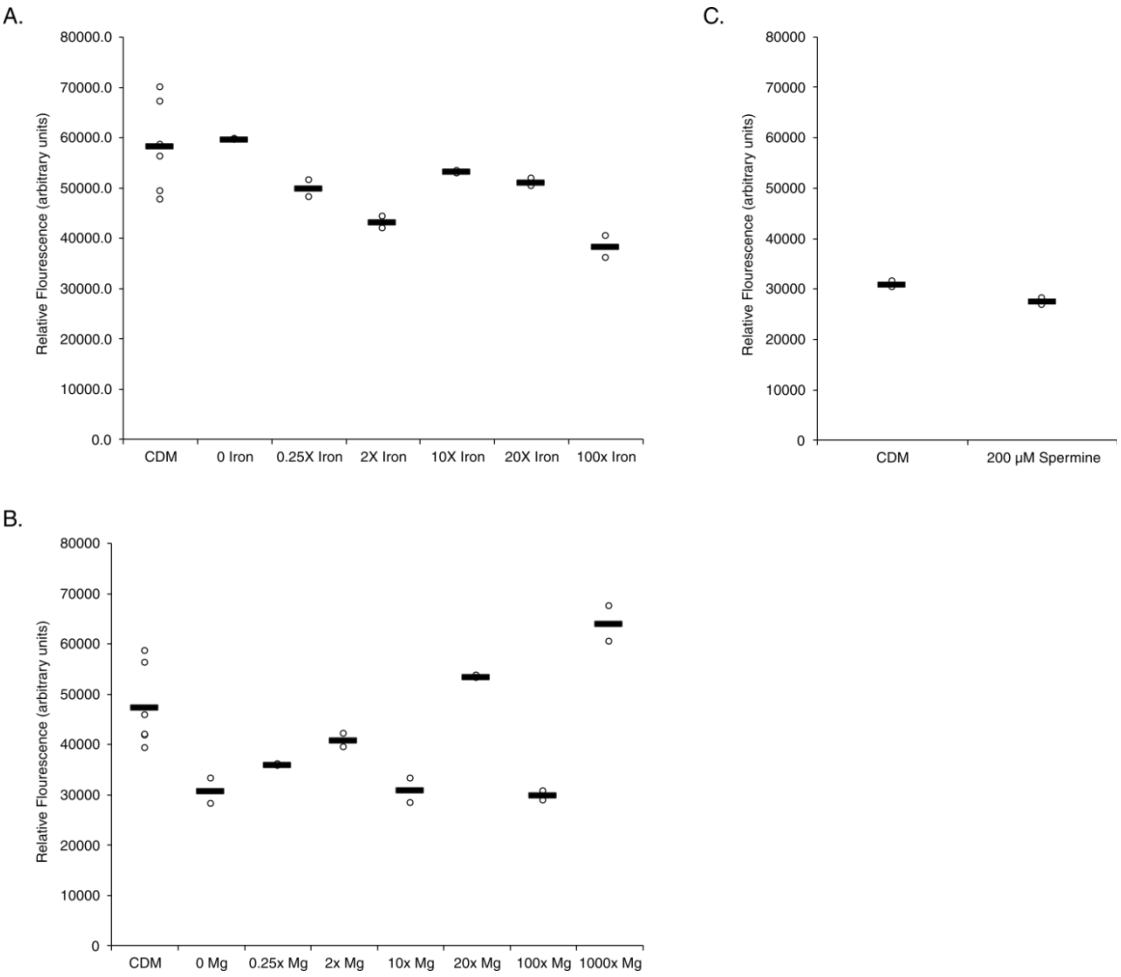

Figure 2. *F. tularensis* cells exposed to different iron, magnesium, or spermine concentrations do not appear to have changes in *rpsU1* production. Relative fluorescence values (y-axis) versus environmental conditions (x-axis) of cells containing fluorescent *rpsU1* reporter construct. Each dot represents one replicate, and horizontal bars indicate the average. (**A**) Cells exposed to CDM with indicated changes in iron concentration. Experiments

were completed in biological duplicate. (**B**) Cells exposed to CDM with indicated changes in magnesium concentration. Experiments were completed in biological duplicate. (**C**) Cells exposed to standard CDM spermine tetrahydrochloride concentration (100.5 µM) or CDM with 200 µM spermine tetrahydrochloride. All were completed once in biological duplicate.

Screening for factors that influence expression of rpsU3

The same conditions were screened for changes in *rpsU3* abundance. Again, significance was determined by at least a two-fold change between the averages of the test and standard conditions. And, again, there was no significant changes in the *rpsU3* abundance when cells were exposed to cold or heat-shock, higher additional salt concentrations, H_2O_2 or UV stress (**Fig 3A, B, C, D**). The conditions tested on *rpsU3* in CDM also showed no significant change in *rpsU3* reported fluorescence in response to changes in iron, magnesium, or spermine concentrations (**Fig 4A, B, C**).

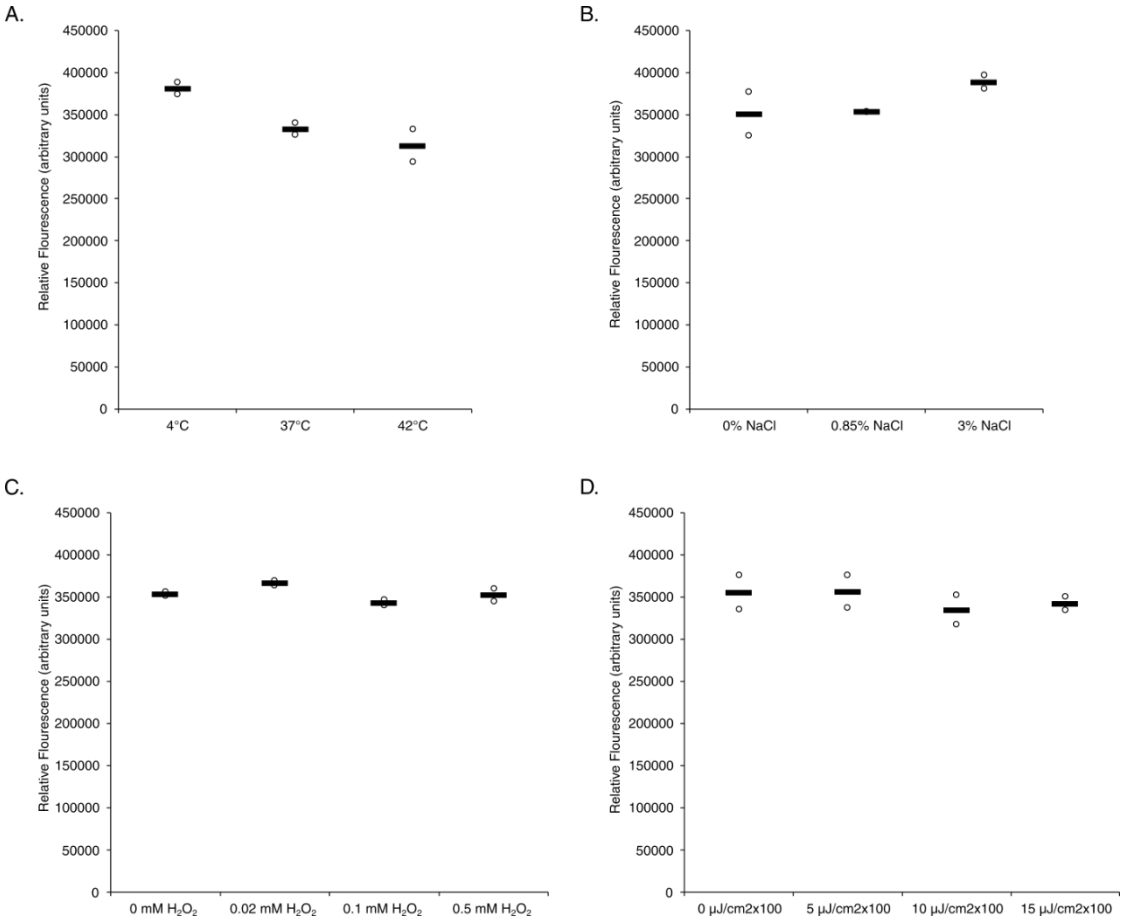

Figure 3. *F. tularensis* cells exposed to different temperatures, salt concentrations, or amounts of oxidative or UV stress do not appear to have changes in *rpsU3* production. Relative fluorescence values (y-axis) versus environmental conditions (x-axis) of cells containing fluorescent *rpsU3* reporter construct. Each dot represents one replicate, and horizontal bars indicate the average. (**A**) Cells exposed to temperatures of 4°C or 42°C as compared to standard growth temperature, 37°C. Experiment was completed in biological duplicate. (**B**) Cells grown in different salt concentrations. 0.5% NaCl and 1% NaCl were tested twice in biological duplicate, the rest were completed once in biological duplicate. (**C**) Cells grown to log-phase and then exposed to indicated concentrations of hydrogen peroxide (H_2O_2). All were completed once in biological duplicate. (**D**) Cells grown to log-phase and then exposed to indicated doses of UV light exposure. All were completed once in biological duplicate.

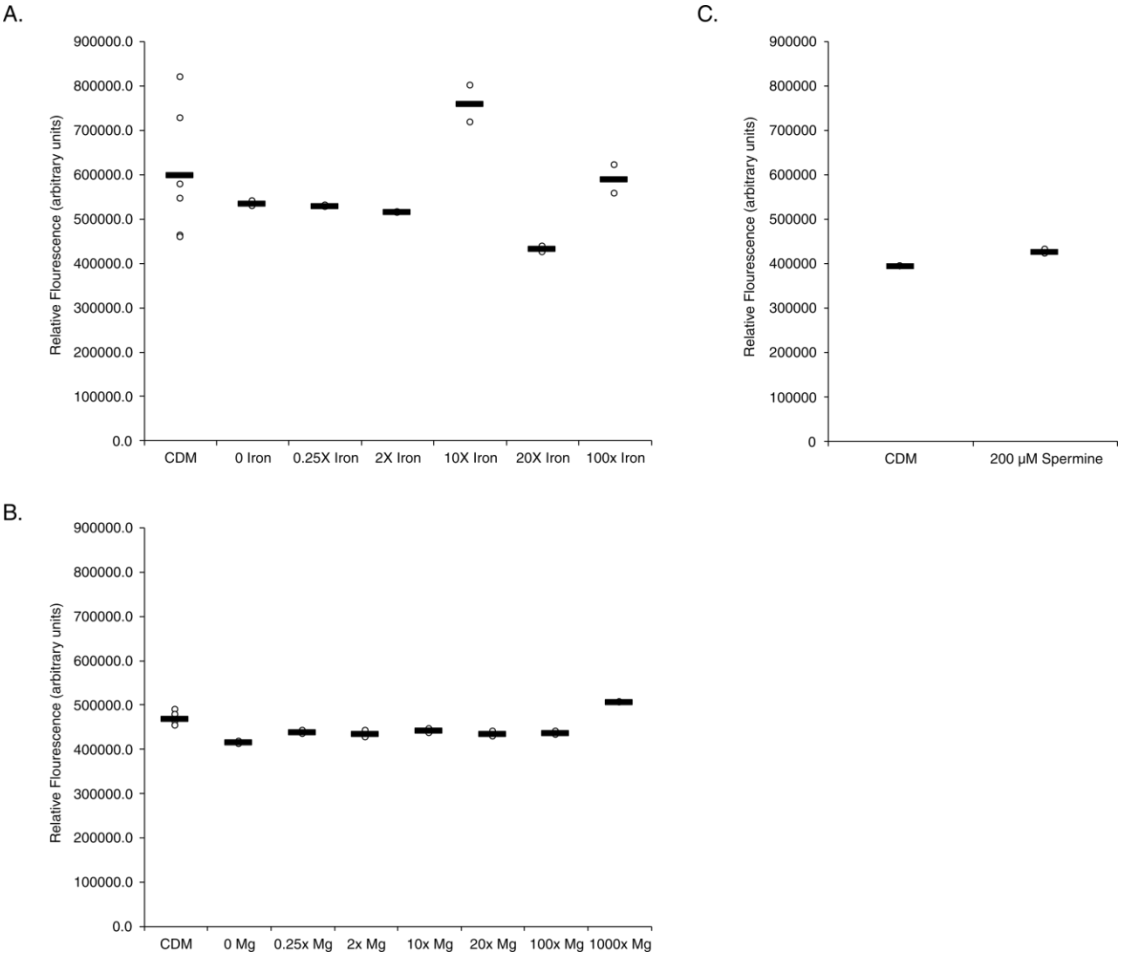

Figure 4. *F. tularensis* cells exposed to different iron, magnesium, or spermine concentrations do not appear to have changes in *rpsU3* production. Relative fluorescence values (y-axis) versus environmental conditions (x-axis) of cells containing fluorescent *rpsU1* reporter construct. Each dot represents one replicate, and horizontal bars indicate the average. (**A**) Cells exposed to CDM with indicated changes in iron concentration. (**B**) Cells exposed to CDM with indicated changes in magnesium concentration. (**C**) Cells exposed to standard CDM spermine tetrahydrochloride concentration (100.5 µM) or CDM with 200 µM spermine tetrahydrochloride. All were completed once in biological duplicate.

Growth in CDM or on CHAH changes bS21-1 abundance

Although cells containing the *rpsU1* fluorescent reporter did not have significant changes in fluorescence when grown in CDM with altered levels of several components, there are differences in fluorescence when comparing cells grown in CDM to those grown in sMHB. Specifically, there was a 2.1-fold

increase in fluorescence when cells with the *rpsU1* reporter construct were grown in CDM compared to sMHB. (**Fig 5A**). Similarly, when grown on solid media (CHAH), cells containing the *rpsU1* reporter construct had a 3.3-fold increase in fluorescence (**Fig 5A**). Conversely, when these cells were grown to stationary phase in sMHB there was a 75% decrease in fluorescence (**Fig 5A**).

To determine if the observed changes in *rpsU1* reporter fluorescence reflect changes in bS21-1 production, we took advantage of cells containing a translational reporter already available in the laboratory. Specifically, these cells encode the *lacZ* gene, encoding ß-galactosidase, immediately downstream of the native *rpsU1* gene on the chromosome. Thus, a β-galactosidase assay can be used to approximate bS21-1 production. We found that growth in both CDM and CHAH produced significantly increased bS21-1, as approximated by β-galactosidase activity, compared to sMHB. However, in cells grown in CDM compared to sMHB, the increase in bS21-1 was only 1.5-fold, compared to the 2.1-fold increase in fluorescence abundance (**Fig 5B**). However, in cells grown in CHAH, the abundance of bS21-1 was increased 3.1-fold (**Fig 5C**).

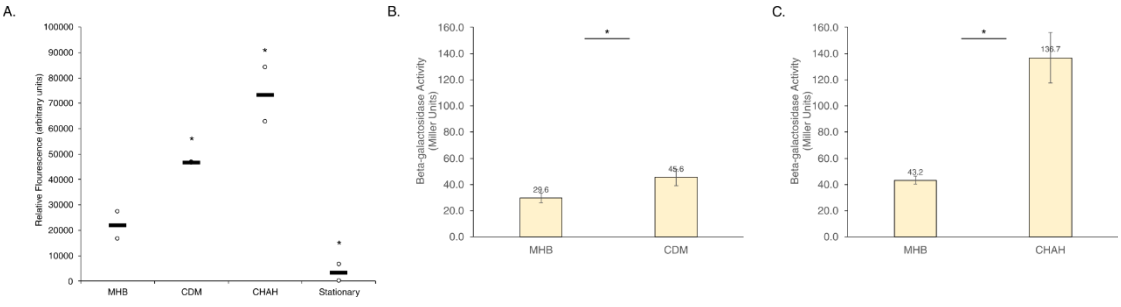

Figure 5. *F. tularensis* cells grown in CDM or on CHAH increases *rpsU1* transcript and protein production. (**A**) Relative fluorescence values (y-axis) versus environmental conditions (x-axis) of cells containing fluorescent *rpsU1* reporter construct. Cells were grown in CDM, on CHAH, or to stationary phase in sMHB, in biological duplicate. Each dot represents one replicate, and

horizontal bars indicate the average. (**B** and **C**) β-galactosidase activity (Miller Units, y-axis) versus environmental conditions (x-axis) of cells containing a single copy *lacZ* fused to *rpsU1,* in the native locus. (**B**) Cells grown in CDM. Completed once in biological duplicate. (**C**) Cells grown on CHAH. Completed twice in biological triplicate. * $p < 0.05$ by two-tailed Student's t test.

Growth in CDM or on CHAH changes bS21-3 abundance

When assessing changes in *rpsU3* transcript and bS21-3 protein abundance in these same conditions, we found an increase in both transcript and protein abundance. However, these increases in fluorescence corresponding to *rpsU3* transcript abundance did not meet our criteria for significance (>/= 2-fold altered). We found a 1.3-fold increase in cells grown in CDM compared to sMHB (**Fig 6A**), and only a 1.6-fold increase in fluorescence of cells grown on CHAH versus grown in sMHB (**Fig 6A**). We did not observe any change in in fluorescence corresponding to *rpsU3* transcript abundance comparing cultures grown to stationary phase versus log phase (**Fig 6A**).

Although these fold-changes did not meet our criteria for significance, the abundance of bS21-3 under these conditions, as approximated by β-galactosidase activity, was tested alongside bS21-1. The translational reporter cells encode the *lacZ* gene, encoding ß-galactosidase, immediately downstream of the native *rpsU3* gene on the chromosome. The change in β-galactosidase activity when grown in CDM was slightly higher than the fold-change in transcript, a 1.5-fold increase, but did not meet our criteria for significance (>/=2-fold) (**Fig 6B**). However, when cells with the translational reporter were grown on CHAH, the β-galactosidase activity increased 2-fold, and was statistically significantly different from cells grown in sMHB (**Fig 6C**).

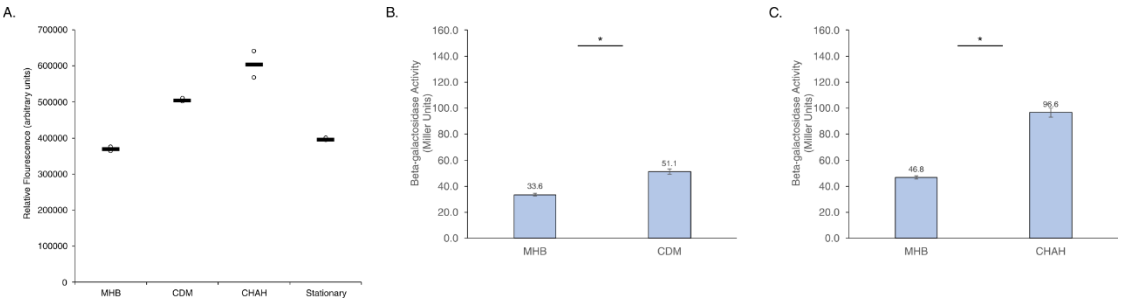

Figure 6. *F. tularensis* cells grown in CDM or on CHAH increases *rpsU3* transcript and protein production. (**A** Relative fluorescence values (y-axis) versus environmental conditions (x-axis) of cells containing fluorescent *rpsU3* reporter construct.) Cells grown in CDM, on CHAH, or to stationary phase in sMHB. Completed in biological duplicate. (B and **C**) β-galactosidase activity (Miller Units, y-axis) versus environmental conditions (x-axis) of cells containing a single copy *lacZ* fused to *rpsU3,* in the native locus. (**B**) Cells grown in CDM. Completed once in biological duplicate. (**C**) Cells grown on CHAH. Completed twice in biological triplicate, a representative example is shown. * $p < 0.05$ by two-tailed Student's t test.

Low pH increases rpsU1 transcript, but not protein abundance

In order to assess the effects of pH on either *rpsU1* or *rpsU3* abundance, cells were grown to log-phase and then transferred into media with altered pH. Assessing the fluorescence of these cells revealed increased *rpsU1* transcript abundance at lower pH (**Fig 7A**). Particularly, while several of the acidic pH values came close to our threshold of a 2-fold change, pH 3.5 was the only acidic culture to cross that mark, at a 2.3-fold increase (**Fig 7A**). Additionally, pH 10 showed a significant decrease in transcript abundance at a decrease of 94% fluorescence (**Fig 7A**).

Unlike the increase observed in transcript abundance at pH 3.5, there was no accordant increase in protein abundance of bS21-1 (**Fig 7B**). In fact, the reporter activity decreased to a level which may indicate that there is no functional β-galactosidase (**Fig 7B**). This was validated multiple times, including

after the media was adjusted to neutral pH, suggesting that the low pH significantly interferes with the β-galactosidase reporter.

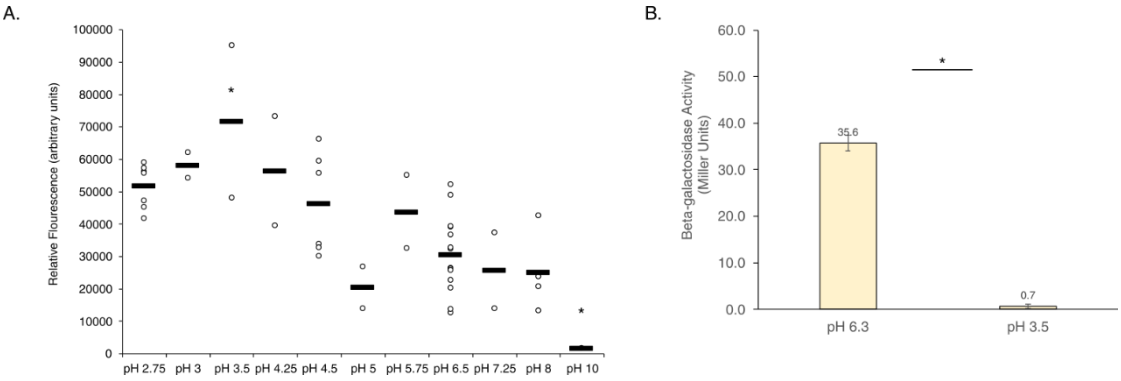

Figure 7. *F. tularensis* cells exposed to low pH increases *rpsU1* transcript, but decreases protein production. (**A**) Relative fluorescence values (y-axis) versus environmental conditions (x-axis) of cells containing fluorescent *rpsU1* reporter construct. Each dot represents one replicate, and horizontal bars indicate the average. Cells grown to log phase and then exposed to indicated pH values. (**B**) β-galactosidase activity (Miller Units, y-axis) versus environmental conditions (x-axis) of cells containing a single copy *lacZ* fused to *rpsU1,* in the native locus. Cells grown to log phase and then exposed to sMHB pH 3.5. Completed three times in biological triplicate, a representative example is shown. * $p < 0.05$ by two-tailed Student's t test.

Changes in pH do not greatly affect rpsU3 transcript abundance, but decrease protein abundance

In contrast, the transcript abundance of *rpsU3* did not seem to strongly react to changes in pH, whether acidic or basic (**Fig 8A**). Only at pH 2.75 was there a large change, at a decrease of 65% fluorescence (**Fig 8A**). Similar to the protein abundance observed at low pH of bS21-1, there is virtually no β-galactosidase activity at pH 3.5 when assessing the bS21-3 *lacZ* reporter (**Fig 8B**).

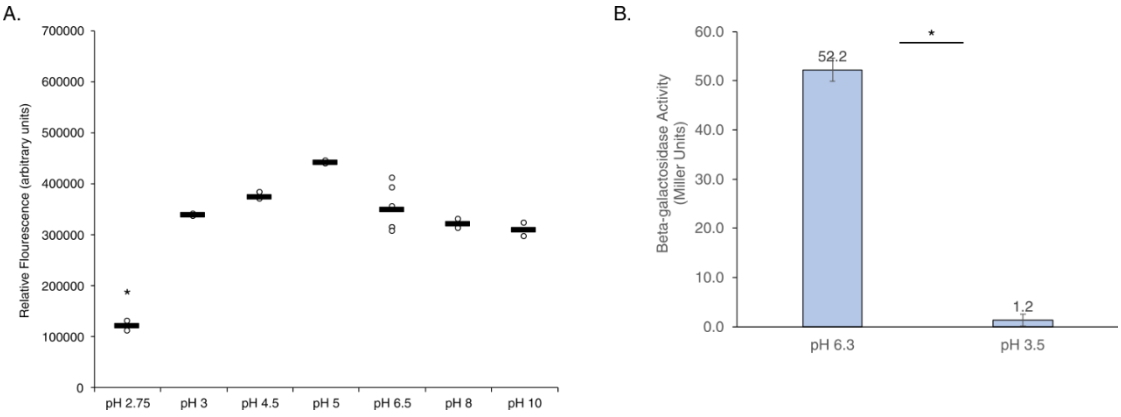

Figure 8. *F. tularensis* cells exposed to different pH do not greatly affect *rpsU3* transcript, but decreases protein production. (**A**) Relative fluorescence values (y-axis) versus environmental conditions (x-axis) of cells containing fluorescent *rpsU3* reporter construct. Each dot represents one replicate, and horizontal bars indicate the average. Cells grown to log phase and then exposed to indicated pH values. (**B**) β-galactosidase activity (Miller Units, y-axis) versus environmental conditions (x-axis) of cells containing a single copy *lacZ* fused to *rpsU3,* in the native locus. Cells grown to log phase and then exposed to sMHB pH 3.5. Completed three times in biological triplicate, a representative example is shown. * $p < 0.05$ by two-tailed Student's t test.

Discussion

In this study we used reporter assays to investigate environmental factors that could lead to differential expression of the ribosomal proteins bS21-1 and bS21-3 in *F. tularensis*. For most of the conditions we examined, we did not observe any significant change in reporter activity. However, based on our transcriptional reporter, there is evidence of increased bS21-1 in response to low pH, growth in CDM, and growth on CHAH. Similarly, we found evidence of increased bS21-3 in cells grown in CDM or on CHAH. We subsequently assessed bS21-1 and bS21-3 production under these conditions using *lacZ*-based translational reporters. We could not validate the increase in bS21-1 in cells exposed to acidic pH, likely due to technical considerations. Finally, we observed an increase in both bS21-1 and bS21-3 in cells grown in CDM or on

CHAH, but the magnitude of increase was lower than observed using the transcriptional reporter assay.

It is important to note that the transcription start sites of the genes encoding bS21-1 and bS21-3 (*rpsU1* and *rpsU3*) are not known, so our transcriptional reporters include both the putative promoter and 5´ untranslated region (UTR). Thus, it is possible that the GFP reporter is subject to both transcriptional and post-transcriptional control. In addition, as *rpsU1* is the second gene in an operon, its promoter corresponds to the sequence upstream of the gene *cspA*. We do not know if other regulatory elements differentially control production of *rpsU1*. Thus, it is possible that the transcriptional reporter activity corresponding to *rpsU1* may more accurately reflect changes in abundance of *cspA.*

With respect to the impact of pH on bS21-1, we observed a disconnect between transcriptional and translational reporter protein abundance (**Fig 7**). It is possible that abundance of *cspA* is increased at low pH and other regulatory mechanisms limit bS21-1 production. However, the cells containing both the bS21-1 and bS21-3 *lacZ* translational reporters had a complete lack of β-galactosidase activity in the low pH condition. This raises the possibility that the low pH externally resulted in sufficiently low internal pH to permanently inactivate the β-galactosidase enzyme.

In considering the different *in vitro* growth media, their complex and undefined nature makes it difficult to determine which factors lead to the changes in bS21 homolog production. While future work could shed light on this

topic, the observed differences were not large and other approaches may be more fruitful.

This study serves as a survey of environmental factors that influence gene expression in *F. tularensis* and were tested for their ability to influence the abundance of ribosomal proteins bS21-1 and bS21-3. While we determined that low pH may impact bS21-1 production and that production of both homologs is altered by different *in vitro* growth media, more questions remain. In particular, it is not clear how these conditions impact protein production nor what other conditions could lead to increased abundance of bS21 homologs. These questions, and how bS21-1 and bS21-3 influence the proteome, will be addressed in future work.

Materials and Methods

Bacterial Strains and Growth Conditions

For standard growth, *F. tularensis* subsp. *holarctica* LVS was grown in Mueller Hinton Broth supplemented with 0.025% iron pyrophosphate, 0.1% glucose, and 2% Isovitalex (sMHB) aerobically shaking at 37°C or on cystine heart agar plates containing 1% hemoglobin (CHAH) at 37°C. As indicated, LVS was grown in Chamberlain's Defined Medium, CDM (Chamberlain, 1965). The selective antibiotic kanamycin (kan) was used to maintain plasmids in *F. tularensis* LVS at 5 µg/mL.

Plasmids

To generate plasmids in which the promoter region of either *rpsU1* or *rpsU3* drive expression of *gfp*, DNA upstream of the first gene in each operon was amplified with a forward primer specifying a 5´ KpnI site and a reverse primer specifying a 5´NotI site. For *rpsU1*, this fragment included the *cspC* start codon and 274 bp upstream. For *rpsU3*, this fragment included the *rpsU3* start codon and 381 bp upstream. These fragments and the plasmid pKR145, which contains the sfGFP gene codon-optimized for expression in *F. tularensis* LVS (Trautmann et al., 2023), were digested with KpnI/NotI and ligated together. This resulted in replacement of the promoter region in pKR145 with either the *rpsU1* or *rpsU3* promoter region, in frame with the respective start codon, yielding pKR153 pF-*rpsU1*-GFP and pKR154 pF-*rpsU3*-GFP.

Strains

To screen environmental conditions that might alter expression of *rpsU1* or *rpsU3*, we electroporated either pKR153 or pKR154 into *F. tularensis* LVS as previously described (Trautmann et al., 2023). For use in the β-galactosidase assay, strains KRLVS28 and KRLVS75 were used. The genotypes of these strains were LVS *rpsU1-lacZ* and LVS *rpsU3-lacZ* respectively. These were previously created using allelic exchanged pEX-based plasmids, pKR34 (pEX_*rpsU1_lacZ*) or pKR38 (pEX_*rpsU3_lacZ*).

Screening for Environmental Conditions - Growth

Changes in temperature, salinity, pH, hydrogen peroxide (H_2O_2) stress, and UV stress were tested in sMHB. To test salinity and pH, cells were grown to early-log phase and transferred to fresh, modified sMHB for an additional 30-60 minute growth period. Alternatively, for temperature, H_2O_2, and UV stress, cultures were grown to mid-log phase, exposed to the stress, and allowed to recover for 30 minutes. sMHB versus CDM was tested by growing cultures to mid-log phase in sMHB or CDM. Changes in iron, magnesium, or spermine concentrations were tested in CDM by altering the recipe to reflect the concentrations tested, and allowing the cultures to grow to mid-log phase.

GFP Assay

After cultures were grown to mid-log phase, 1 - 4mL were pelleted and resuspended in 1xPBS. In technical triplicate, 250 uL was plated in a clear 96-well plate and the OD_{600} was read by the SpectraMax® iD3 Multi-Mode Microplate Reader. 200 uL of the sample was then transferred to a black 96-well plate and fluorescence was measured (RFU). All fluorescence values were

made relative to the OD_{600}; and relative fluorescence from LVS containing an empty vector (pF) in 1xPBS was subtracted from the relative fluorescence of our strains in the test conditions. The threshold for changes to be considered significant is a two-fold or greater change from fluorescence of control cells.

β-Galactosidase Assay

Assays were performed essentially as previously described (Trautmann, et al., 2023). Briefly, cultures were grown to early mid-log (OD_{600} 0.3 – 0.35) in triplicate. Growth was halted by placing cultures on ice for at least 30 minutes before cells were added to tubes containing Z-buffer supplemented with β-mercaptoethanol. 0.1% SDS and chloroform were added, cultures were vortexed, then allowed to come to 28°C for 10 minutes prior to the addition of the substrate O-nitrophenyl-β-galactopyranoside (ONPG). Reactions were stopped upon reaching a yellow color, OD_{420} 0.6-0.9, using sodium carbonate (Na_2CO_3).

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CHAPTER 4

SUMMARY AND CONCLUSIONS

Chapter 2 Summary

As described in Chapter 2, the results of our transcriptomic analysis revealed that any of the three bS21 homologs of *F. tularensis* can repress the abundance of the *rpsU2* operon transcript. However, bS21-1 and bS21-3 were not found to regulate their own expression, indicating that autoregulation of bS21-2 is unique among the three homologs.

We subsequently used the sequences upstream of *rpsU2* and translational *lacZ* reporter fusions to further investigate the *cis*-factors that lead to bS21-2 autoregulation. Using qRT-PCR, we found that these reporters accurately recapitulated the regulation revealed by the transcriptomic data. In particular, the abundance of our reporter transcripts was several fold higher in cells lacking bS21-2 than in wild-type cells. Additionally, it was shown that the 5′ UTR of the *rpsU2* transcript is sufficient to lead to this change in transcript abundance. Yet, when measuring the relative protein production, β-galactosidase assays showed that in cells lacking bS21-2, contrary to the increase in transcript abundance, reporter protein activity (and presumably bS21-2 protein abundance) did not similarly increase.

Secondary structures predicted in the 5′ UTR of *rpsU2* were tested as a potential site of regulatory activity. Translational reporters using the 5′ UTR of *rpsU2* fused to GFP were used to assess the contribution of these stem loops to both the transcript and protein abundances of *rpsU2* and bS21-2. Our qRT-

PCR results indicated that the first 38 nucleotides of the leader sequence are not required for regulation of transcript abundance. In addition, GFP assays also indicated that these nucleotides did not result in significant changes in fluorescence, suggesting that they were not required for regulation of translation of bS21-2.

Finally, we assessed how bS21-2 influences the stability of the *rpsU2* transcript. We found that the half-life of the *rpsU2* transcript was increased in cells lacking bS21-2 in multiple experiments. This suggests that bS21-2 destabilizes its own transcript. However, due to variability among the replicates, this change in transcript half-life was not statistically significant in any of the experiments. Thus, further testing would be required to confirm whether bS21-2 destabilizes its own transcript as the mode of autoregulation.

While this study examined some interesting aspects of regulation of the bS21 homologs, there are many questions remaining.

Chapter 3 Summary

In Chapter 3, I screened different growth conditions to identify stimuli that lead to altered abundance of bS21-1 or bS21-3. In particular, I used GFP reporter fusions to screen for potential changes in transcript abundance corresponding to either bS21-1 or bS21-3. While I tested eleven conditions, I identified only two in which there may be major altered abundance of bS21-1 and/or bS21-3.

When compared to cells grown in our standard culture media, supplemented Mueller-Hinton Broth (sMHB), cells grown on cystine heart agar

plates with hemoglobin (CHA-H plates) had an increase in reporter activity corresponding to both bS21-1 and bS21-3. This was also true of the cells grown in Chamberlain's chemically defined liquid medium (CDM). Both findings were replicated using reporters of bS21-1 and bS21-3 protein abundance, cells in which the *lacZ* gene is present on the chromosome directly after the native *rpsU1* or *rpsU3* gene, respectively.

I also found that cells with the plasmid-based *gfp* reporter for bS21-1 had increased reporter gene expression in low pH MHB. This suggests that low pH could lead to an increased abundance of the *rpsU1* transcript. However, these results could not be validated in cells containing the chromosomally-encoded bS21-1 *lacZ* reporter construct. In fact, there was nearly no reporter gene activity (ß-galactosidase activity) after cells were incubated for one hour in low pH.

The discordance between these reporters may be explained by a few options. First, the *gfp* reporter construct to assess bS21-1 production may more accurately reveal regulation of the first gene in the operon, the cold shock family protein, *cpsC*. It is possible that *cspC* is induced in low pH conditions, but that other regulatory mechanisms prevent additional production of bS21-1 (and thus, production of ß-galactosidase from the translational reporter). However, it seems more likely that the low pH conditions inactivated the ß-galactosidase enzyme. In particular, I also found that cells with the bS21-3 *lacZ* reporter construct had no reporter activity when tested in the same low pH condition.

Together, I found that *F. tularensis* cells grown in different growth media appear to have increased production of bS21-1 and bS21-3, and low pH may induce production of bS21-1. However, much remains to be found about how production of these ribosomal proteins are controlled.

FURTHER QUESTIONS

1. Does bS21-2 definitively affect the stability of its own transcript, and is this a direct interaction?

In Chapter 2, I explored the *cis*-encoded factors which lead to the regulation of the *rpsU2* transcript by its own protein. Specifically, I established that the 5′ UTR of *rpsU2* is sufficient to lead to regulation by bS21-2. This led me to investigate whether or not bS21-2 was impacting the stability of its own transcript. In multiple replicates of this experiment, the mRNA corresponding to the *rpsU2* operon had increased stability in wild-type cells compared to cells lacking bS21-2. However, these differences were not statistically significant. This could have either resulted from stability simply not being the factor affected by bS21-2, or it could be due to a need to optimize the protocol to reduce variability amongst replicates. Thus, to determine if stability actually plays a role in the regulation of the *rpsU2* transcript, it would be necessary to repeat the stability assay described in Chapter 2.

If stability leads to the autoregulation by bS21-2, then it would be interesting to determine the exact mechanism by which bS21-2 controls mRNA abundance. Given that the 5′ UTR of *rpsU2* is sufficient for regulation, and that there are many examples in which a transcript is mediated by an interaction (directly or indirectly) with the leader sequence, it would be informative to determine what elements of the 5′ UTR are necessary for the bS21-2-mediated changes in mRNA abundance. As described in Chapter 2, the two stem-loops at the 5´ end of the 5′ UTR are not necessary for regulation by bS21-2. To identify which elements of the 5′ UTR are necessary, we could make similar reporters with

various mutations or truncations of the 5′ UTR and assess their relative transcript abundance in cells with and without bS21-2 to identify mutants that are no longer responsive to bS21-2.

We could further determine whether bS21-2 exerts its effects by making direct contact with its own transcript using an electrophoresis mobility shift assay (EMSA). Specifically, we would purify bS21-2 and combine it with either its own transcript or a control (an mRNA unaffected by bS21-2). These samples are separated by size using gel electrophoresis. If bS21-2 stably binds directly to its own mRNA, we expect to see a band indicating that bS21-2 is forming a complex with its own mRNA transcript. In the control condition, we expect to see no evidence of bS21-2 complexing with the mRNA transcript. This would allow us to determine if bS21-2 binds directly to mRNA transcripts . In particular, we would test the ability of bS21-2 to bind to the *rpsU2* 5′ UTR compared to the *tul4* 5′ UTR, and any modified *rpsU2* 5′ UTRs that are unresponsive to bS21-2.

2. What controls the expression of bS21 protein abundance?

Apart from the transcriptional regulation of *rpsU2* discussed in Chapter 2, I also demonstrated that there is a disconnect between *rpsU2* transcript abundance and bS21-2 protein abundance. This suggests that there is another factor regulating the translation of the *rpsU2* transcript. A few methods could be used to elucidate these other factor(s).

We could take an unbiased genetic approach to identify repressors of *rpsU2* translation. First, we would construct a strain in which, at the native locus, GFP is inserted after the first six amino acids of the *rpsU2* gene, replacing the rest of

the gene. Then, we would utilize mariner transposon-led mutagenesis of the GFP reporter construct to indicate genes of interest. Presumably, were the transposon to interrupt a gene whose product represses bS21-2 then the GFP abundance would increase, and this will be observed as colony color changes. Individual mutants of interest will then be further verified through fluorescence readings. Subsequently, mutants with higher abundance of bS21-2 will be sequenced and analyzed to reveal potential regulatory proteins, particularly by determining where the transposon inserted in the genome. The interrupted gene would then, presumably, encode regulatory proteins.

In a biochemical approach, we could attempt to identify proteins that directly bind the *rpsU2* 5′ UTR. Specifically, we would obtain a synthesized version of the *rpsU2* 5′ UTR containing a 3´ biotin tag. We would incubate this with cell lysates and isolate any RNA-protein complexes by immunoprecipitation using streptavidin beads. Associated proteins will be identified using mass spectrometry, generating a list of proteins that interact with the bS21-2 transcript and may regulate the protein abundance of bS21-2. These can be further characterized through standard genetic knockout studies.

3. What genetic elements influence the production of bS21-1 and bS21-3?

In Chapter 3, I screened a number of growth conditions to identify significant changes in both transcript and protein abundance of bS21-1 and bS21-3. However, most of the environments screened did not result in any statistically or biologically significant changes in either or both transcript and protein abundance.

Another way to identify factors controlling expression of these bS21 homologs would be to take a genetic approach. In particular, I propose taking a transposon mutagenesis approach to screen for genes important for bS21 homolog production, using GFP as a reporter. Specifically, I would generate cells in which there are fusions of bS21-1 or bS21-3 with GFP, by adding *gfp* in-frame to the 3´ end of the native *rpsU1* or *rpsU3* gene on the chromosome. We would then mutagenize these cells and screen for colonies which produce more or less fluorescence. The subsequent analysis and interpretation would be similar as described above in future direction 2. In this case, we expect to identify genes that function to regulate bS21-1 or bS21-3 production.

It is important to mention that we do not know how strong the fluorescent signal for bS21-1-GFP or bS21-3-GFP would be. If, in otherwise wild-type cells, the signal is very low, we expect to only be able to detect increases in fluorescence. In this case, because this strategy relies on inactivation of gene products by transposon mutagenesis, we would expect to identify repressors of bS21-1 or bS21-3. We would be unlikely to find positive regulators of bS21-1 or bS21-3 expression (whose inactivation would lead to less fluorescence). However, this remains to be determined.