CHAPTER 1

LITERATURE REVIEW AND INTRODUCTION

REVIEW OF THE LITERATURE

Translation initiation is a target for regulation

Regulation of gene expression through by modifying translation is a rapid way for bacteria to adapt to changing environmental or nutrient requirements. The ribosome is the highly conserved molecular machine that translates mRNA into protein and consists of two subunits composed of ribosomal RNAs (rRNAs) and ribosomal proteins (r-proteins). The complex process of protein synthesis is carried out through the coordinated actions of the ribosomes, tRNAs, and a number of additional factors. This process can be broken down into three major phases: translation initiation, elongation, and termination. While some regulation can occur during elongation, particularly in stress conditions (Taylor et al., 2013), initiation is the rate-limiting step of protein synthesis and the target of most regulatory mechanisms (Duval et al., 2015). As a brief overview, translation initiation begins with the formation of the 30S initiation complex, during which the Shine-Dalgarno (SD) sequence of mRNA can bind to the anti-Shine-Dalgarno (ASD) sequence of the 16S rRNA in the small ribosomal subunit (30S). The SD-ASD interaction increases the efficiency of translation initiation and assists in correctly positioning the initiation codon in the ribosome. Initiation factors help the anticodon on the initiating fMet-tRNA interact with the start codon of the mRNA in the P site. Subsequently, initiation factors are released and the large ribosomal subunit (50S) joins to form the 70S initiation

16S rRNA, bS21, bS6 and bS18 (Jha et al., 2020) that result in sequestration of the ASD. This provides a rationale for why most genes in *F. johnsoniae* do not have Shine-Dalgarno sequences – transcripts cannot interact with the ASD because of the sequestration by r-proteins. Loss of bS21 results in increased translation from the *rpsU* mRNA, which has an extended Shine-Dalgarno (McNutt et al., 2023). Thus, bS21 autoregulates its own protein production through direct binding interactions with the ribosome (Jha et al., 2020; McNutt et al., 2023). This mechanism may differ in other classes of bacteria, in part because the interactions between bS21 and the ASD in *F. johnsoniae* that lead to sequestration of the ASD is in the C-terminal region of bS21, which is variable and lineage-specific. In particular, bS21 homologs in Gammaproteobacteria have not been found to, and are not expected to, sequester the ASD. Indeed, many species including our model organism, *Francisella tularensis*, have many mRNAs with strong SDs.

Notably, a large number of *rpsU* homologs have been found in bacteriophage genomes. Over 1,300 bS21 homologs were found in viral genomes, infecting a wide range of bacterial clades (Al-Shayeb et al., 2020; Mizuno et al., 2019). Metatranscriptomic analyses identified bS21 transcripts associated with late stages of viral replication (Chen et al., 2022). At least one bS21 homolog was found to be encoded by a cultivated pelagiphage, *Pelagibacter* phage HTVC008M, and the purified viral bS21 protein was able to be incorporated into *E. coli* ribosomes (Mizuno et al., 2019). This is likely possible because bS21 is one of the last-assembled r-proteins and is easily

may suggest post-transcriptional regulation – the study observing the protein-level differences may not detect transcript-level differences if there is another layer of regulation. Additionally, in *F. novicida*, loss of *hfq* led to decreased transcript abundance of *pdpA* and *pdpB* (Chambers & Bender, 2011), which conflicts with the previous study in LVS (Meibom et al., 2009). The differing results between *F. tularensis* and *F. novicida* may suggest species-specific differences in the Hfq regulon. Regardless of the specific impact on FPI genes, Hfq plays an important role in stress response in *F. novicida* and in virulence in mouse infection assays for *F. tularensis* clinical isolates (Chambers & Bender, 2011; Meibom et al., 2009).

As Hfq often works coordinately with sRNAs, it is worth pointing out that very few sRNAs have been identified in *F. tularensis.* In additional to identification of well-known sRNAs (4.5S rRNA, tmRNA), researchers found two novel sRNAs, referred to as FtrA and FtrB, which impact the transcript abundance of several genes (Postic et al., 2010). However, none of the impacted genes are on the FPI, and FtrA and FtrB do not play an important role in virulence (Postic et al., 2010). A distinct sRNA, FtrC, impacts transcript abundance of the hypothetical protein FTL_1293 independently of Hfq. Overexpression of FtrC does impact intramacrophage growth and infection in mice, though the mechanism is not understood (Postic et al., 2012). Hfq and sRNAs undoubtedly merit further research to uncover the specific role they play in regulating gene expression and virulence, particularly given the conflicting Hfq results and scarcity of sRNAs identified.

HU

HU (histone-like protein U) is a highly conserved bacterial protein that functions similarly to a eukaryotic histone by binding DNA in a sequence-independent manner and altering its structure to affect gene expression and DNA replication (Pettijohn, 1988; Verma et al., 2023). In *F. tularensis*, the genes that encodes HU, *hupB*, is essential for replication in macrophage and pathogenicity in mouse models (Stojkova et al., 2018). The FPI genes and *pigR* were found to both less abundant in the *hupB* mutant at the transcript and protein level, although the mechanism by which HU positively regulates their expression is not clear (Stojkova et al., 2018).

OsrR

A recently described transcriptional regulator called OsrR appears to play a role in regulating gene expression in response to oxidative stress. OsrR not only regulates the oxidative response, but leads to global changes in transcription and virulence. In cells lacking OsrR, transcripts of *pdpE* and *iglJ* are decreased significantly upon exposure to the superoxide-generating compound menadione, which causes oxidative stress. Further, the *osrR* deletion mutant leads to decreases in intramacrophage growth and pathogenicity in mice (Marghani et al., 2021).

While *F. tularensis* survives in diverse environments and requires coordinated regulation of the T6SS to replicate in macrophages and infect hosts, there is limited knowledge of regulatory networks in this bacterium. The regulation of FPI transcription is well-studied and there are a few recent advances in identifying novel virulence factors that regulate transcription (OsrR

structure of the *F. johnsoniae* ribosome reveals three bS21 residues interact with the ASD: K51, Y54, and I55. Neither K51 or Y54 are conserved in the *F. tularensis* bS21 homologs (Figure 3A). I55 is partially conserved, as bS21-3 also has an isoleucine in that position, and bS21-1 and bS21-2 have a valine, which has similar properties to isoleucine (Figure 3A). Because of these differences, we do not expect the *F. tularensis* bS21 homologs to sequester the ASD; there may be another mechanism that allows bS21 homologs in *F. tularensis* to impact gene expression.

In addition to the 16S rRNA, bS21 also interacts with the r-proteins bS18 and uS11 in the *E. coli* ribosome (Berk et al., 2006). At least four bS21 residues are likely to interact with bS18: two are conserved in all of the *F. tularensis* bS21 homologs (C22, K19), one is a partially conserved (I3), and one is not conserved (K4). Ten bS21 residues may interact with uS11, spanning most of the bS21 protein. Again, some of these residues are completely conserved in the *F. tularensis* bS21 homologs, some are partially conserved, and some are not conserved (Berk et al., 2006). These comparisons draw attention to the importance of solving a structure of the *F. tularensis* ribosome, to determine where bS21 homologs interact with rRNA and other proteins, how these interactions might differ between the three homologs, and whether these may suggest functional differences amongst bS21 homologs.

The evolutionary history of bS21 homologs is not known

While Chen et al. (2020) looked at the divergent evolution of bS21 homologs in Bacteroidia and phages, no publications to our knowledge have

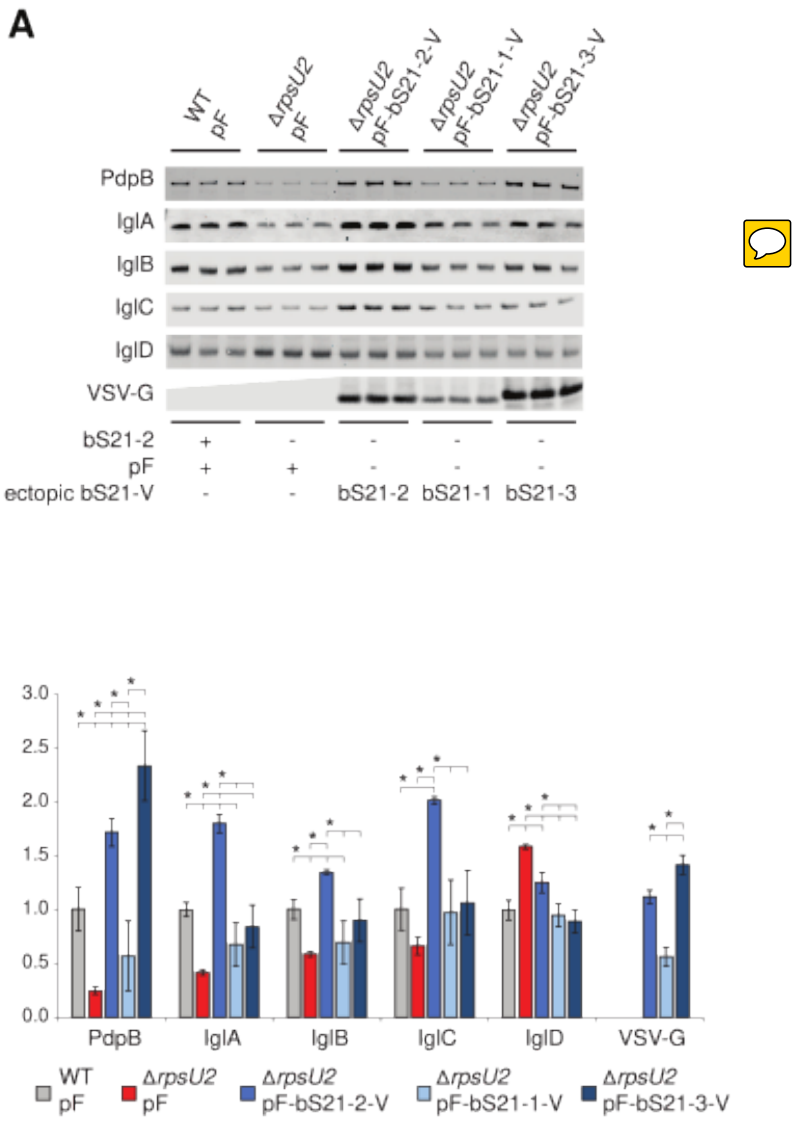

Figure 3. bS21-2 impacts T6SS protein abundance. A. Immunoblot analysis of indicated T6SS protein abundance. Cells either contained (wild-type) or lacked (Δ*rpsU2*) bS21-2 and either an empty vector control (pF) or a vector ectopically expressing VSV-G-tagged bS21 homologs (pF-bS21-1-V, pF-bS21-2-V, or pF-bS21-3-V). Immunoblot against VSV-G was included to demonstrate production of VSV-G-tagged bS21 homologs. **B.** Quantification of immunoblots from (A). Band intensities for each protein were normalized to total protein per well on the membrane. Error bars represent 1 SD. Experiments were repeated at least twice and data from a representative experiment are shown. Lines above bars indicate statistical comparison among groups by t-test. Asterisk indicates group to which all other groups are compared, if horizontal line connects to line above group, *$p <$ 0.05 using Benjamini-Hochberg correction.

rpsU mRNA and mRNAs engineered to have a strong SD (McNutt et al., 2023). These studies unambiguously demonstrate that ribosomes lacking bS21 have altered specificity for particular mRNAs in translation initiation, providing evidence that bS21 functions as a bona fide regulator of gene expression (McNutt et al, 2023).

Francisella tularensis, a human pathogen that requires a type VI secretion system (T6SS) to cause disease, encodes three distinct homologs of bS21. We have shown that all three homologs can be incorporated into ribosomes, and loss of one of the homologs, bS21-2, leads to changes in protein abundance of most T6SS-encoding genes that cannot be explained by changes in transcript abundance or protein stability. Loss of bS21-2 also results in defective intramacrophage replication in cells that can be complemented by restoration of bS21-2, but not the other two homologs. This indicates that bS21-2 specifically governs virulence genes, including those that encode the T6SS (Trautmann & Ramsey, 2022).

While our results support a model in which bS21 proteins in *F. tularensis* regulate gene expression at the level of translation, it is clear that bS21 homologs in Gammaproteobacteria exert their effects in a manner distinct from bS21 homologs in Bacteroidia. The bS21 proteins in *F. tularensis* and *F. johnsoniae* differ significantly at the amino acid level, particularly in the variable C-terminal (Jha et al., 2020). In Bacteroidia, there are multiple conserved C-terminal residues required for bS21 to interacts with the ASD that are not conserved in other bacterial

This work demonstrates that bS21-2 exerts its effects on protein synthesis in a leader sequence-dependent manner and is validated in a subset of bS21-2-responsive 5´ UTRs (**Fig 2**). While loss of bS21-2 results in altered abundance for about 160 proteins (Trautmann & Ramsey, 2022), we expect that changes in protein abundance for at least some of these may not be due leader-sequence dependent effects, but rather due to downstream or secondary effects. For example, bS21-2 may directly impact synthesis of proteins that influence the abundance of other proteins. Several proteases and peptidases were found to be differentially abundant in cells lacking bS21-2. Thus, proteins like those encoded by FTL_0881 and FTL_0215 may have altered abundance in bS21-2 mutant cells due to changes in the abundance of proteases or protein processing genes.

In our search for an element responsible for leader sequences to be responsive to bS21-2, we found that ideal SD sequences prevent responsiveness. These leader sequences with perfect SDs also lead to much higher translation. This suggests to us that perfect SD-ASD complementarity leads to such efficient translation that any contribution of bS21-2 to translation are minor and effectively masked. It is perhaps unsurprising that other regulators of translation, such as H-NS in *E. coli*, similarly function to regulate translation of mRNAs with imperfect SDs (Park et al., 2010).

While we were unsuccessful at identifying a common element across all bS21-2 responsive leader sequences, we were able to hone in on the 6 nt sequence in the *mraY* 5´ UTR that leads to bS21-2 responsiveness, GACUCU. It

intramacrophage replication assays revealed that bS21-2 is important for virulence. Loss of bS21-2 led to an intramacrophage growth defect that could be restored by complementation with bS21-2, but not with expression of bS21-1 or bS21-3.

Altogether, the research described in Chapter 2 showed us that bS21-2 modulates expression of many genes at the protein level, including key virulence genes encoding the T6SS, and promotes intramacrophage replication. These data support our model that bS21-2 specifically impacts expression of some genes, either directly or indirectly, during translation initiation. Combined with other recent findings of bS21 impacting translation of subsets of mRNA in different bacterial species, our work supports a new model of bS21-driven gene regulation at the level of translation. Further research into this field may reveal that other types of heterogeneous ribosomes can impact different stages of translation to rapidly alter bacteria's proteome. However, the mechanism by which bS21-2 impacts some genes in *F. tularensis* remained an open question.

CHAPTER 3 SUMMARY

In the next chapter of my research, we investigated the mechanism that leads to altered protein abundance for bS21-2-responsive genes. We validated that there is an increase in abundance RNA-binding protein Hfq in cells lacking bS21-2. Together with the fact that Hfq is known to impact FPI gene expression, this raised the possibility that impacts of bS21-2 on the FPI could be due to Hfq. If so, would expect that Hfq acts as a negative regulator of all FPI genes during protein synthesis. While we validated that Hfq negatively regulates the FPI, we