

RESEARCH STRATEGY SIGNIFICANCE

Elucidating control of virulence genes in *F. tularensis*

Francisella tularensis is the causative agent of tularemia, is a highly infectious bacterial intracellular pathogen, and is considered a potential bioweapon^{1,2}. The ability of *F. tularensis* to cause disease is dependent on its ability to survive within host cells, particularly macrophage³. A virulence factor that is absolutely essential for intramacrophage growth of *F. tularensis* is its type VI secretion system (T6SS)⁴⁻⁸. Significant work has elucidated complex mechanisms that lead to transcriptional regulation of T6SS genes, encoded on the Francisella Pathogenicity Island (FPI). Transcription of FPI genes is increased by interaction of two RNA polymerase-associated transcription factors, MglA and SspA, with the small molecule ppGpp and the DNA-binding transcription factor PigR⁹⁻¹⁴. These factors interact with, and promote transcription of, FPI promoters due to the presence of a specific sequence element, the PigR-responsive element (PRE), in these promoters^{14,15}. However, while these findings represent significant progress in understanding how FPI genes are regulated transcriptionally, very little is known about the translational regulation of these key virulence genes. The studies proposed here would increase our basic understanding of *F. tularensis* pathogenesis by elucidating post-transcriptional regulation of the FPI genes and other virulence genes.

A ribosomal protein controls translation of virulence genes

We have recently identified a novel regulator of virulence gene expression, the small subunit ribosomal protein (r-protein) bS21-2¹⁶. The model we propose to explain how bS21-2 exerts its effects is illustrated in **Fig 1**. The bS21-2 protein is one of three homologs of bS21 encoded in the *F. tularensis* genome. Only one molecule of bS21 is incorporated into a single ribosome and we have demonstrated that each bS21 homolog can be incorporated into actively-translating ribosomes. Additionally, analysis of bulk purified *F. tularensis* ribosomes revealed the presence of multiple bS21 homologs, together demonstrating that ribosomes in *F. tularensis* are heterogenous with respect to bS21¹⁶.

One of the bS21 homologs, bS21-2, is uniquely important for virulence of *F. tularensis* and governs expression of critical virulence genes. When cells lack bS21-2, they produce less T6SS proteins. This reduction is not due to reduced transcript abundance of FPI genes, but of reduced translation of mRNAs (**Fig 2**)¹⁶. Cells lacking bS21-2 also have intramacrophage replication defects. Although the other bS21 homologs can partially or fully complement T6SS protein production, only bS21-2 can restore intramacrophage replication¹⁶. Thus, while it is clear that one homolog of a ribosomal protein, bS21-2, is specifically important for *F. tularensis* virulence and controls virulence gene expression post-transcriptionally, how it exerts its effects is unknown. Understanding how the homologs of this ribosomal protein influence *F. tularensis* virulence gene expression is the primary focus of this study. Our global hypothesis is that *F. tularensis* incorporates different bS21 homologs into ribosomes to control gene expression during translation (**Fig 1**). We expect our results will extend to other bacteria that encode either a single or multiple bS21 homolog and advance our understanding of post-transcriptional gene regulation.

Connecting ribosome composition and gene regulation

It is common for bacteria to encode multiple homologs for at least one ribosomal protein, which raises the possibility of heterogeneity in ribosome composition¹⁷. Heterogenous ribosomes have been found in multiple organisms and can also be the result of sequence-specific differences in rRNA, post-transcriptional, or post-translational modifications (reviewed in¹⁸). We have demonstrated that ribosomes in *F. tularensis* are heterogenous with respect to bS21 content¹⁶. The presence of ribosome heterogeneity raises two key questions: (1) How is ribosome composition regulated? and (2) What is the functional impact of ribosome heterogeneity?

In specific cases, regulation of bacterial ribosome composition is well-understood. Some bacteria, including mycobacteria and *Bacillus subtilis*, encode r-proteins paralogs that either do or do not coordinate zinc (reviewed in¹⁹). When in zinc-replete conditions, these organisms 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. While this well-described mechanism permits coordinated control of the appropriate r-protein paralogs, there is a significant gap in our knowledge with respect to how cells regulate

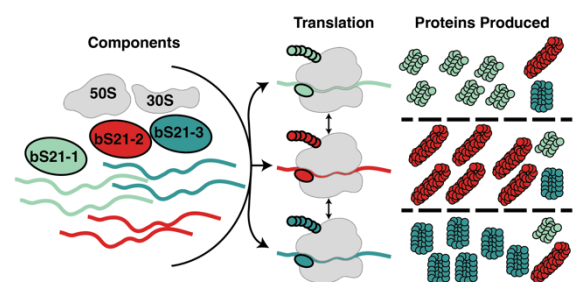


Figure 1. Model of how ribosome heterogeneity due to multiple bS21 homologs influences gene expression in *F. tularensis*. During initiation of translation, any one of three bS21 homologs (in Components) can be assembled into 70S ribosomes. Ribosomes with specific bS21 homologs preferentially initiate translation of specific mRNAs and bS21 homologs can be exchanged between assembled 70S ribosomes (Translation). Resulting protein abundance reflects preferential translation initiation (Proteins Produced).

production of multiple r-protein homologs that do not coordinate a divalent cation, such as the bS21 homologs in *F. tularensis*. None of the bS21 homologs in *F. tularensis* are predicted to coordinate divalent cations and there are no studies examining how the appropriate amount of bS21 is maintained in *F. tularensis* cells.

The consequences of ribosome heterogeneity on protein synthesis remain unclear. The intriguing idea that modifying ribosome composition leads to functional differences, allowing them to become “specialized,” has been proposed many times and is the topic of many reviews^{18,20–25}. However, much of the evidence for specialized ribosomes is derived from studies of eukaryotic ribosomes. The evidence for specialized bacterial ribosomes to date is extremely limited and sometimes controversial. For example, toxin-mediated ribosome modification (reviewed in²⁰) leading to altered activity has been challenged²⁶. Other cited examples of ribosomes altered in composition and function include either synthetically engineered ribosomes²⁷ or those isolated under non-physiologically relevant conditions (exposure to antibiotic and induction of a particular gene²⁸). Ribosomes that incorporate different r-protein paralogs clearly change in composition and are thought to allow dynamic control of intracellular zinc concentrations^{19,29}. It is less clear how zinc-related differences in r-protein composition impact function; there is accumulating evidence there are changes to protein synthesis, yet the molecular mechanism remains undefined^{30,31}. Arguably the clearest and most physiologically-relevant evidence of ribosomes with altered protein synthesis activity due to altered composition can be found in a recently published paper describing the role of bS21 in an entirely different phylum, Bacteroidia (formerly Bacteroidetes), further described below³².

bS21 is a poorly-understood conserved regulatory ribosomal protein

The small ribosomal subunit protein bS21 (previously known as S21, encoded by the *rpsU* gene), is a small r-protein that is not required for ribosomal assembly, codon recognition, or peptide bond formation. Instead, its primary role appears to be in translation initiation^{33–35}. While bS21 has been lost in multiple lineages, its amino acid sequence has been conserved in those lineages that have retained it. It appears that bS21 is essential in some organisms, but is not strictly required for protein synthesis *in vitro*^{17,36}. This makes bS21 unique among ribosomal proteins: it is highly conserved, sometimes essential, but not critical for ribosome integrity or protein synthesis. Thus, we hypothesize that bS21 serves a regulatory function in translation initiation.

Unfortunately, bS21 is not found in all ribosome structures, either because some bacteria do not encode it or because, as one of the last proteins to be incorporated into the 30S subunit, stringent washing conditions can dissociate bS21 from the ribosome during purification^{37,38}. However, structures of ribosomes containing bS21 reveal it is ideally positioned to interact with mRNA leader sequences during translation initiation, close to the Shine-Dalgarno (SD) helix near the RNA exit channel^{39–41}. Our structural understanding of possible interactions between bS21 and leader sequences is limited because, to the best of our knowledge, there is no ribosome structure with bS21 and an RNA template with a sufficiently long leader sequence. However, there is evidence that the presence of bS21 is necessary for translation of some, but not all, mRNAs by *E. coli* ribosomes and we have used translational reporters to identify bS21-mediated differences in translation initiation in *F. tularensis* (Fig 2)³⁵. Consistent with its potential to function as a regulatory, rather than structural, component of the ribosome, bS21 can be easily exchanged among assembled ribosomes^{37,42}.

In at least one bacterial phylum, Bacteroidia, it is clear that bS21 can function to regulate protein synthesis³². In discussing this mechanism, it is important to remember that in most bacteria, including γ -proteobacteria like *F. tularensis*, the mRNA-encoded SD sequence base-pairs with the anti-Shine-Dalgarno (ASD) encoded within the 3' end of the 16S rRNA⁴³. This interaction is thought to position the mRNA correctly in the ribosome for initiation and strong SD-ASD interactions generally lead to increased protein synthesis. However, in Bacteroidia, mRNAs rarely have SD sequences – even though the ASD encoded by the 16S rRNA is conserved⁴⁴. The first structure of a Bacteroidia ribosome (from *Flavobacterium johnsoniae*) reveals that the ASD sequence is inaccessible to mRNAs as the result of interactions with r-proteins bS18, bS6, and the single bS21 homolog⁴⁵. Critically, the mRNA encoding bS21 contains an extended consensus SD sequence and *F. johnsoniae* ribosomes lacking bS21 have increased synthesis of mRNAs with SD sequences, including the mRNA encoding bS21 itself³². These results demonstrate that the presence or absence of bS21 alters ribosome activity in *F. johnsoniae*. Specifically, distinct classes of ribosomes preferentially initiate translation on distinct mRNAs, those either with or without SD sequences. While these exciting findings reveal that bS21 functions in a regulatory capacity, this mechanism depends on sequestration of the ASD by the C-terminal end of bS21 and this specific C-terminal of bS21 is only conserved in Bacteroidia⁴⁵. Therefore, the mechanism by which other bS21 homologs govern gene expression is unknown.

In bacteria outside of Bacteroidia, inactivation of bS21 leads to specific phenotypes, strongly suggesting that bS21 plays a regulatory role in many other organisms, including multiple pathogens. In *Listeria monocytogenes*, inactivation of bS21 is linked to stress resistance and altered transcript abundance and in *B. subtilis*, bS21 loss results in biofilm and motility defects^{46–49}. Loss of bS21 in *Staphylococcus aureus* is associated with increased

resistance to the antibiotics daptomycin and vancomycin^{50–55}. Both *Burkholderia pseudomallei* and *F. tularensis* encode multiple bS21 homologs and in both organisms, one homolog is important for virulence^{56,57}. Finally, consistent with the potential for bS21 to become co-opted to influence gene expression, multiple phage-encoded bS21 genes have been identified and at least one phage-encoded bS21 can be incorporated into *E. coli* ribosomes, outcompeting the native (endogenous) bS21^{58–60}. Together, these findings suggest that bS21 is important for regulating specific pathways, possibly by exerting its effects during translation initiation.

The ribosome as a drug target: the untapped potential of heterogeneity

Many clinically-used antibiotics are protein synthesis inhibitors that target the ribosome. Studies of ribosome structure have provided a deep understanding of how the ribosome functions, how drugs can inhibit it, and how antibiotic resistance arises. Most of this extensive research has used model organisms like *Escherichia coli* and fewer studies have focused on ribosomes in pathogens⁶¹. While the function of the ribosome is universally conserved, there are significant differences in ribosome structures among bacterial species and, given the common occurrence of ribosome heterogeneity, possibly even within the same species.

The study of ribosome heterogeneity and its consequences offer several avenues for future antimicrobial development. The first is via the identification of species-specific ribosome-targeting drugs. Although highly conserved, changes in ribosome structure between species may differ enough to allow species specificity of ribosome inhibitors^{62,63}. The second avenue is the select inhibition of ribosome subpopulations. If a particular class of ribosomes is critical for virulence, as we describe below, inhibitors specifically targeting these ribosomes would effectively create anti-virulence compounds⁶⁴. Because ribosome heterogeneity is common, this general approach may be applicable to multiple pathogens, not just those with multiple bS21 homologs.

Rigor of prior research

The proposed work depends on the following information: (1) bS21 controls gene expression and (2) distinct bS21 homologs in *F. tularensis* control expression of different genes. With respect the first, the body of literature that is described above and includes studies performed in many different bacteria, suggest that the conserved function of bS21 is to regulate translation initiation. Our recently published work contributes to this body of knowledge and describes how a bS21 homolog in *F. tularensis* influences gene expression and particularly expression of key virulence genes¹⁶. Additionally, the recent study of the function of bS21 in *F. johnsoniae* demonstrated that bS21 can control gene expression explicitly using rigorous biochemical and genetic methods³².

With respect to the distinct functions of bS21 homologs in *F. tularensis*, we have demonstrated that one homolog in particular is key for virulence and that the three homologs are not functionally identical¹⁶. Loss of bS21-2 results in reductions of T6SS proteins, and cells lacking bS21-2 are defective for intramacrophage growth. While production of another homolog, bS21-3, can restore T6SS protein levels, this homolog cannot compensate for loss of bS21-2 in intramacrophage survival. These results demonstrate that bS21-1, bS21-2, and bS21-3 are not interchangeable and have distinct impacts on gene expression in *F. tularensis*. These foundational data have been peer-reviewed and published¹⁶.

INNOVATION

Conceptual: Our data suggest *F. tularensis* controls virulence gene expression in a novel manner – by altering ribosome composition and activity to preferentially translate subsets of mRNAs. Our understanding of gene regulation at the level of translation in bacteria largely focuses on factors influencing mRNA rather than the translational machinery⁶⁵. Further validating that ribosome composition plays a role in regulated protein synthesis would represent a significant shift in our understanding of gene regulation. Most studies of bacterial ribosome structure have been performed on model organisms; this would be the first analysis of *F. tularensis* ribosomes and one of only a few from an actual pathogen. The molecular mechanism by which the r-protein bS21 impacts bacterial phenotypes is poorly-understood and there are very few studies examining its role in translation^{32,34,35}; these would represent the first studies identifying the roles of individual bS21 homologs in the same cell. These would also be the first studies to examine the molecular mechanisms by which ribosomes containing different bS21 homologs influence virulence, gene regulation, and ribosome structure.

Methodological: During these studies, we will utilize proven methodologies to address conceptually innovative questions. Several of these methodologies represent state-of-the-art techniques, including high-throughput sequencing approaches (ribosome profiling and RNA-Seq) and cryo-electron microscopy. While the methods we propose are not inherently original, their combined use to address our hypotheses is innovative. Lastly, ribosome profiling is a relatively new technique and because it has not been performed in *F. tularensis*, its use constitutes a novel approach.

APPROACH

In the below aims, we propose to further investigate the role of bS21 in control of virulence genes in *F. tularensis*. The experiments outlined here use the Live Vaccine Strain (LVS) of *F. tularensis*, which is used in Dr. Ramsey's laboratory following Biosafety Level 2 protocols. This strain is attenuated for virulence in humans and is considered an exempt strain^{66,67}. Because the genes encoding bS21 and potential regulatory sequences are highly conserved among *F. tularensis* strains, we believe our studies will be informative to understanding gene regulation in *F. tularensis* broadly, including in highly pathogenic type A strains such as SCHU S4.

Aim 1: Assess how *F. tularensis* bS21 homologs control virulence and global gene translation.

Rationale and Preliminary Results. We have demonstrated that bS21-2 influences translation of specific mRNAs in *F. tularensis*. In particular, we found that loss of bS21-2 leads to post-transcriptional changes in the abundance of about 160 proteins, including type VI secretion system (T6SS) proteins critical for virulence¹⁶. Because the location of bS21 in the ribosome is ideally positioned to interact with 5' untranslated regions (UTRs) during translation initiation, we speculated that bS21 homologs may influence translation initiation to regulate protein abundance (**Fig 1**). In particular, we hypothesized that bS21 homologs may interact directly with 5' UTRs of specific transcripts and influence the rate of translation initiation.

To test this hypothesis, we generated translational reporters with 5' UTRs fused to a reporter gene (**Fig 2A**). These 5' UTRs included a control (corresponding to a gene unaffected by bS21-2, *tul4*) and several 5' UTRs corresponding to genes controlled by bS21-2. Transcription of these reporters is controlled by a promoter unaffected by bS21-2 (**Fig 2A**)¹⁶. Translational reporter fusions were introduced into cells with or without bS21-2 and assessed for reporter activity. Consistent with our published proteomic data, we found that the 5' UTRs of regulated genes (such as FPI-encoded *pdpA*, encoding the T6SS protein PdpA and *mraY*) fused to reporters lead to less reporter activity in cells lacking bS21-2 (**Fig 2B, C**, and additional not shown). This demonstrates that these leader sequences are sufficient to lead to altered translation in a bS21-2-dependent manner. We further investigated what leader sequences are required for these effects and found that 5' UTRs containing ideal SD sequences are not responsive to the presence of bS21-2 (**Fig 2B** and additional not shown). These results indicate that bS21-2 positively influences translation only in the absence of strong SD-ASD interactions. Finally, in the 5' UTR of a particular mRNA, we identified a specific 6-nucleotide sequence necessary for bS21-2-mediated increased translation (**Fig 2C**).

These studies provide evidence that bS21-2 influences translation of particular mRNAs due to specific 5' sequences, possibly via specific interactions between bS21-2 and 5' UTRs. They lead us to further hypothesize that bS21 homologs influence gene expression at the level of translation genome-wide. This is consistent with our work in which we demonstrated that different bS21 homologs have distinct effects on gene expression¹⁶. The experiments proposed in this aim will comprehensively identify transcripts that are preferentially translated by ribosomes containing specific bS21 homologs and elucidate the mRNA-specific determinants that allow this control. We expect these results will not only provide further information about regulation of *F. tularensis* virulence genes but will also provide insight into how heterogenous ribosomes influence gene expression.

Research Design

Aim 1a. Determine how bS21 homologs influence translation genome-wide

We will use ribosome profiling to assess and compare translation by ribosomes containing specific bS21 homologs in a sensitive and unbiased manner, genome-wide^{68,69}. Ribosome profiling is a powerful technique that leverages high-throughput sequencing (HTS, also referred to as next-generation sequencing, NGS) to provide global quantitative information about protein synthesis⁶⁸. Essentially, ribosome profiling requires halting actively translating ribosomes (polysomes, stopped by temperature and/or drug treatment), digesting RNA not protected by the ribosome ("footprinting" using micrococcal nuclease),

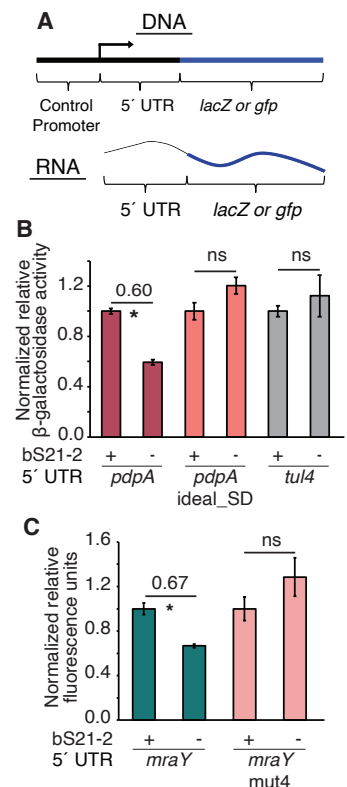


Figure 2. Specific leader sequences lead to bS21-2 controlled translation. **A.** Translational fusion design. The *tul4* promoter drives expression of 5' UTRs fused to reporter genes. **B** and **C.** Relative reporter activity for indicated translational fusions in cells with (+; WT) or without (-; $\Delta rpsU2$) bS21-2 in biological triplicate. Error bars are 1 SD. * $p < 0.05$ by t-test. Experiments were repeated at least twice and representative data are shown. **B.** Translation of reporters with the *pdpA* 5' UTR is increased in cells with bS21-2, but not if modified to include an ideal SD (*pdpA* ideal_SD). The *tul4* 5' UTR serves as a control. **C.** Translation of reporters with the *mraY* 5' UTR is increased in cells with bS21-2, but not if modified to change a specific 6 nt sequence (*mraY* mut4).

purification of the ribosome-protected mRNA fragments, and manipulation of these mRNA fragments into DNA suitable for NGS⁷⁰. We anticipate this approach will be successful given the combination of Dr. Gregory's expertise in ribosome biochemistry, Dr. Ramsey's expertise in NGS library creation and analysis, and our successful isolation of *F. tularensis* polysomes¹⁶.

To compare translation by ribosomes with

distinct compositions, we will perform ribosome profiling on several samples: (1) all the ribosomes in the cell to assess total translation, (2) ribosomes containing only bS21-1, (3) ribosome containing only bS21-2, and (3) ribosome containing only bS21-3. In order to accomplish this goal with appropriate rigor and minimally perturb overall ribosome abundance, we will immunoprecipitate each class of ribosomes from the same cells using epitope-tagged versions of bS21 homologs encoded at their native genomic loci. We have already demonstrated that epitope-tagged bS21-2 can complement loss of the native bS21-2 and we have immunoprecipitated 70S ribosomes using an epitope-tagged bS21-2 (**Fig 3**)¹⁶. This approach will ensure that all cells have biologically relevant amounts of total ribosomes and still provide homolog-specific information about translation.

While performing ribosome profiling experiments, we will be following published protocols and monitoring multiple parameters to ensure experimental success⁷⁰; we will only continue after achieving robust results at each intermediate step. Specifically, to assess the quality of our cell lysates and footprinting, we will fractionate ribosomes from lysates not treated by micrococcal nuclease (which should yield 70S ribosomes and polysomes, similar to published profiles¹⁶) and compare this to treated lysates (in which the polysomal mRNA should be digested, yielding a robust peak for 70S ribosomes). Library preparation requires highly efficient ligation between isolated RNA and a DNA adapter; performing a control ligation with a synthesized RNA adapter will allow us to assess efficiency of ligation. During library preparation, rRNA is a significant contaminant and specific oligonucleotides can be used to deplete contaminating rRNA sequences. To identify the most common rRNA contaminants in our *F. tularensis* ribosome profiling libraries, an initial library will be created without contaminant removal and sequenced at low depth. Subsequent libraries will be prepared using rRNA subtraction. Libraries will be sequenced on an Illumina NovaSeq 6000. The resulting sequencing data will be analyzed using available programs such as cutadapt to remove adapter sequences⁷¹, bowtie2 to align sequences to the genome⁷², and RiboProfiling to analyze ribosome footprints⁷³. We will be assessing parameters such as periodicity of read alignments (because the ribosome translocates three nucleotides at a time, this periodicity is a hallmark of a bona fide translated region⁷⁴) and lack of reads in intergenic regions.

While performing ribosome profiling to assess how much translation of a given transcript is occurring, it is also important to know how much of each transcript is present. RNA-Seq is a well-established technique used to measure transcript abundance genome-wide⁷⁵. Dr. Ramsey has used RNA-Seq in *F. tularensis* with success^{14,16,76} and we will use similar protocols to create next-generation sequencing (NGS) libraries from isolated RNA.

As with the ribosome profiling experiments, we will sequence the RNA-Seq libraries on an Illumina NovaSeq 6000. Our analysis pipeline will include removing adapter sequences with cutadapt⁷¹, aligning to the *F. tularensis* genome with bowtie2⁷², counting reads using HTSeq-Count⁷⁷, and performing differential expression analysis with DESeq2⁷⁸. We will validate a subset of our results using quantitative reverse transcription PCR (qRT-PCR).

By performing ribosome profiling coupled with RNA-Seq on all ribosomes, we will gain genome-wide information about the relative amount of translation for all *F. tularensis* mRNAs. The profiling of specific ribosome populations will allow us to determine which mRNAs are preferentially translated by ribosomes containing specific bS21 homologs. We will validate a subset of our results using mass spectrometry methods, using a metabolic labeling approach for quantification and to achieve maximum sensitivity.

Aim 1b. Identify sequences necessary and sufficient for bS21 homologs to control translation

We have already used translational fusion reporter assays to investigate what sequences in mRNA leaders allow control by bS21-2 (**Fig 2**). We will continue to use this approach to validate a subset of the observations made in Aim 1a. Additionally, we will extend and expand upon these studies to define sequences that are necessary and sufficient to allow control by each of the bS21 homologs. In particular, we will use bioinformatics analyses of our results from Aim 1a to predict features of mRNAs regulated by each bS21 homolog. We can then use reporter constructs, introduced into cells with different bS21 homologs, to systematically alter and examine how these features contribute to bS21-mediated translation, analogous to results in Fig 2.

Given that the above reporter assays are performed in *F. tularensis* cells, we cannot exclude the possibility that other cellular factors, in addition to bS21 homologs, influence translation for particular mRNAs. To address

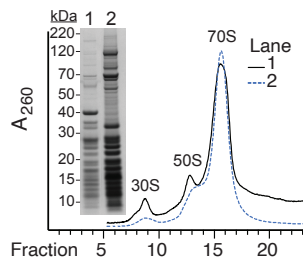


Figure 3. Purified *F. tularensis* ribosomes analyzed by SDS-PAGE and sucrose gradient fractionation. Lane 1: proteins immunoprecipitated with bS21-2-TAP. Peptides corresponding to all r-proteins except bL36 (MW ~4 kDa) were identified by mass spectrometry. Lane 2: ribosomes purified using a sucrose cushion¹⁶. Sucrose fractionation of ribosomes purified by both methods reveals density correspond to 70S particles.

this and complement our *in vivo* reporter assays, we will also take an *in vitro* approach. Specifically, we will use a commercially available kit that includes all the components needed for transcription and translation of a template, but to which ribosomes can be added (NEB PURExpress Δ Ribosome kit). As we have cells that contain only a single bS21 homolog (see Table 1), we can purify ribosomes containing a specific bS21 homolog. Using both *in vivo* and *in vitro* reporter assays will allow us to determine if the regulation of translation for a particular mRNA is due to direct effects by ribosomes or possibly by indirect effects of other post-transcriptional regulators.

Aim 1 Potential pitfalls and alternate approaches

We will carefully consider and evaluate epitope tags for immunoprecipitation of bS21 homologs prior to performing ribosome profiling. Specifically, we will use small tags unlikely to interfere with function (e.g., VSV-G, V5, HA) fused to the C-terminal end, although bS21 in *E. coli* has been tagged with a significantly larger epitope (MBP) with no effects on cell growth (personal communication and ⁷⁹). We will evaluate tagged homologs and only take advantage of those that do not influence cellular growth. However, it is likely that there will be different efficiencies of bS21 homolog immunoprecipitation by different epitope tags. Because we have shown that bS21-2 is the most abundant homolog in wild-type cells, we will prioritize epitope tags that provide the most efficient pulldown for bS21-1 and bS21-3. Additionally, if these differences lead to insufficient data for a given homolog, we will repeat the ribosome profiling after switching epitope tags, a previously used successful approach³⁰.

Use of ribosome profiling, rather than whole-cell proteomics, is the ideal approach to address our model because our interest is focused on the relative amount of translation for a given mRNA by a particular type of ribosome. In contrast, whole-cell proteomics provides information relevant to steady-state protein stability. Furthermore, although quantitative proteomics have advanced significantly, ribosome profiling can provide data that is more quantitative and detailed at the genome-wide scale⁸⁰.

Results from Aim 1a are expected to reveal which transcripts are preferentially translated by ribosomes containing different bS21 homologs. A caveat is that we will be unable to ascertain if ribosomes with a particular bS21 homolog interact with an additional factor to control translation of particular mRNAs. This will be addressed using the *in vitro* validation proposed in Aim 1b. To prevent additional factors from co-purifying with ribosomes, we will use stringent washing conditions to remove associated proteins. We will also assess ribosomes for purity by mass spectrometry. Finally, our results from Aims 2 and 3 are expected provide relevant information about other potential regulators and mechanisms of regulation.

Aim 1 Timeline. We expect work on Aim 1a will begin in year 1 and extend through year 2. Aim 1b will initiate in year 2 and extend through year 5.

Aim 1 Summary

We have demonstrated that a bS21 homolog influences translation of particular mRNAs, including those key for virulence, and that the three bS21 homologs in *F. tularensis* have distinct effects on gene expression. In the work proposed, we will elucidate the role of each bS21 homolog in genome-wide translation using ribosome profiling and subsequent validation using reporter assays. Specifically, we will assess which mRNAs are preferentially translated by ribosomes containing specific bS21 homologs and subsequently identify what elements of those mRNAs allow bS21-specific control. These comprehensive results will provide key insights into the molecular mechanism(s) underlying bS21-mediated control of gene expression and significantly advance our understanding of *F. tularensis* virulence gene regulation. We additionally expect them to shed light on why *F. tularensis* encodes three bS21 homologs. Critically, our findings will provide novel and exciting information about how ribosome heterogeneity impacts gene expression and virulence, which are likely to extend to other bacteria and bS21-encoding phage.

Aim 2: Investigate regulation of bS21-2 production.

Rationale and Preliminary Results. One of the three *F. tularensis* bS21 homologs, bS21-2, controls T6SS protein production and is important for intramacrophage survival¹⁶. However, the factors that influence production of the bS21 homologs themselves have not been studied. Here we provide evidence that production of bS21-2 is regulated by all three bS21 homologs, but the mechanism by which these homologs exert their effects is unknown. The goal of this aim is to uncover the regulatory mechanisms that lead to production of the *F. tularensis* bS21 homolog critical for virulence, bS21-2.

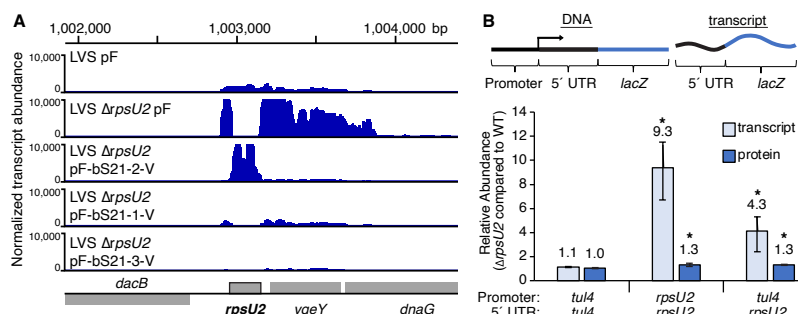


Figure 4. The bS21-2 transcript (*rpsU2*) is influenced by all bS21 homologs but transcript doesn't correlate with protein abundance. **A.** RNA-Seq of wild-type (WT) cells or cells lacking bS21-2 ($\Delta rpsU2$) and either empty vector (pF) or vector with indicated bS21. Y-axis is truncated at 10,000 for clarity. A subset of data is published¹⁶. Experiments were performed in biological triplicate, representative data shown. **B.** Indicated translational fusions assessed for reporter transcript and protein abundance, comparing amounts in cells lacking bS21-2 ($\Delta rpsU2$) with wild-type cells (WT). Transcript abundance measured by qPCR, protein abundance by β -galactosidase activity. Error bars are 1 SD. * $p < 0.05$ by t-test. Experiments were performed in biological triplicate, repeated at least twice, representative data shown.

Ribosomes are abundant and energetically costly, so cells must carefully regulate ribosomal protein (r-protein) production. It is typical for operons encoding multiple r-proteins to be regulated by one of the first r-proteins to bind rRNA in assembly (primary binding r-proteins), as these proteins can recognize and bind specific mRNA features that are structurally analogous to their binding sites on rRNA⁸¹. Regulation of r-protein production has not been studied in *F. tularensis* and there are several unique and unexplored elements about regulation of bS21 homologs in *F. tularensis* in comparison with other systems. First, to the best of our knowledge, this is the first report of autogenous regulation of bS21; *E. coli* encodes only a single bS21 homolog which is not autogenously regulated^{81,82}. Secondly, 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 found in operons with other primary binding r-proteins⁸³. Finally, in *F. tularensis*, the cell must coordinate appropriate production of three different bS21 homologs. As described in the Significance section, production of alternate r-protein paralogs is well-described for r-proteins which do or do not coordinate zinc; to the best of our knowledge, there are no studies of regulated production of r-protein homologs which do not coordinate zinc. Thus, there are no established models to understand how production of bS21 homologs in *F. tularensis* are controlled.

We have found that all three bS21 homologs in *F. tularensis* influence the amount of RNA encoding bS21-2. Specifically, we have found that transcript abundance of the operon encoding bS21-2, the *rpsU2* operon, is significantly increased when cells lack the gene encoding bS21-2 (*rpsU2*) (**Fig 4A**)¹⁶. However, upon ectopic expression of any of the three bS21 homologs, the abundance of the *rpsU2* operon transcript returns to wild-type or lower levels (**Fig 4A**)¹⁶. Thus, all three bS21 homologs influence the abundance of RNA encoding bS21-2 and YqeY (which is encoded downstream of *rpsU2*). We have recapitulated these results using a reporter assay in which the promoter and 5' UTR of *rpsU2* are fused to *lacZ* and integrated in single copy into the chromosome at a neutral site (the Tn7 site)⁸⁴. In cells without bS21-2, there is significantly more *lacZ* transcript (~9-fold) compared to cells with bS21-2; note that there is no significant difference when using a translational fusion for a control gene promoter and 5' UTR (**Fig 4B**). Together, these results demonstrate that bS21-2 and the other bS21 homologs are repressors of *rpsU2* operon transcript abundance, although the mechanism by which they exert their effects is unknown.

Steady-state levels of RNA, as determined by RNA-Seq and qRT-PCR, reflect both the synthesis and degradation of a given transcript. Which of these processes (or if both) are influenced by bS21 homologs to control *rpsU2* operon transcript abundance is not known. Additional reporter fusion experiments demonstrate that the 5' UTR of *rpsU2* is sufficient for increased transcript abundance in the absence of bS21-2, suggesting that these effects are at least partially the result of post-transcriptional regulation (**Fig 4B**). Since the 5' UTR of *rpsU2* is sufficient to increase transcript abundance in the absence of bS21-2, we hypothesize that mRNA stability, mediated by the 5' UTR of the *rpsU2* transcript, is a key factor in the control of *rpsU2* transcript abundance. In fact, we have preliminary data indicating that the *rpsU2* operon transcript is more stable in cells lacking bS21-2 (wild-type half-life: 2.7 min; $\Delta rpsU2$ half-life: 15.4 min). Accordingly, we hypothesize that interactions between bS21 homologs and some element(s) of the 5' UTR lead to decreased stability of the *rpsU2* operon transcript when bS21 homologs are abundant.

While all three bS21 homologs influence the abundance of the *rpsU2* operon mRNA, there are additional unknown factors that control the amount of bS21-2 protein. Despite the increase in *rpsU2* operon transcript in cells without bS21-2, we do not observe a corresponding increase in protein. In particular, cells without bS21-2 have 6-fold more *yqeY* transcript, which corresponds to the gene immediately downstream of *rpsU2*¹⁶. Despite this increase in transcript abundance, there is no significant change in YqeY protein abundance, as measured by DIA mass spectrometry¹⁶. The discordant relationship between transcript abundance and protein abundance is also apparent in the translational fusion reporter assay, in which the promoter and 5' UTR of *rpsU2* are fused to *lacZ*. When compared to wild-type cells, cells lacking bS21-2 have ~9-fold more *lacZ* transcript, yet there is a

less than 1.5-fold increase in β -galactosidase abundance (~30%; **Fig 4B**). This demonstrates that there are additional unknown mechanisms regulating protein synthesis of genes in the *rpsU2* operon.

Not only is bS21-2 important for expression of key virulence genes and intramacrophage replication, production of this r-protein is controlled by multiple factors. The proposed experiments in this aim will elucidate the mechanisms of control for this r-protein critical for virulence. We expect these results will not only provide further information about regulation of *F. tularensis* virulence but may also shed light on the regulation of other non-zinc coordinating r-proteins.

Research Design

Aim 2a. Determine how bS21 homologs lead to lower levels of the bS21-2 transcript

To dissect the mechanisms that lead to control of bS21-2 production, we will assess the contribution of cis- and trans- elements that control *rpsU2* operon transcript abundance. Because the *rpsU2* 5' UTR is sufficient to increase transcript abundance in the absence of bS21-2, we hypothesize that the 5' UTR mediates *rpsU2* operon transcript stability and thus abundance.

To determine the elements of the 5' UTR that are necessary to regulate *rpsU2* transcript abundance, we will use reporter fusion assays. Specifically, we will create mutant versions of the *rpsU2* 5' UTR fused to reporter genes, as in Fig 4, and test their ability to promote increased transcript abundance in cells lacking bS21-2 using qRT-PCR. Secondary structure predictions of the 5' UTR suggest the presence of two highly stable stem-loops at the 5' end of the transcript. It is possible that these stem-loops play a role in modulating transcript stability, so we will start our analyses by truncating the 5' UTR to exclude those loops. We will generate and test additional mutations, including longer truncations and mutations to disrupt the stem-loop structures and restore them, as appropriate. We expect these analyses to identify the element(s) of the 5' UTR that lead to decreased stability in the presence of bS21-2 (and conversely, increased stability in the absence of abundant bS21 homologs).

We will also assess the role of bS21 homologs in repression of the steady-state levels of *rpsU2* transcript. We hypothesize that bS21 homologs directly interact with the *rpsU2* 5' UTR to influence transcript stability. To directly test this possibility, we will perform electrophoresis mobility shift assays (EMSAs), combining purified bS21 homologs and labeled RNA corresponding to the *rpsU2* 5' UTR. We will include controls such as RNA corresponding to an unaffected 5' UTR, titrating unlabeled *rpsU2* 5' UTR, and any mutant versions of the *rpsU2* 5' UTR that are not sensitive to the presence or absence of bS21-2 as discovered above. If our hypothesis is correct, we expect to identify a shift in *rpsU2* 5' UTR mobility in the presence of bS21-2 but not in control conditions, providing strong evidence that bS21-2 directly interacts with the *rpsU2* 5' UTR to influence transcript stability. We will repeat these experiments with each of the other *F. tularensis* bS21 homologs. To extend our experiments to heterologous bS21 homologs, we will assess the ability of bS21 homologs from other species (such as those from *E. coli* or *F. johnsoniae*) to repress steady-state *rpsU2* transcript and subsequently test their ability to interact with the *rpsU2* 5' UTR in EMSAs. These experiments will allow us to identify elements of the bS21 homologs important for directly interacting with the *rpsU2* 5' UTR.

Aim 2b. Identify factors that influence translation of the bS21-2 encoding transcript

While transcript and protein abundance are generally strongly correlated, the effects of post-transcriptional regulation can decouple this relationship. In the case of the *rpsU2* operon, we have observed a dramatic increase in transcript abundance in the absence of bS21-2, but not a corresponding increase in protein abundance (**Fig 4**)¹⁶. These results can be recapitulated using a reporter assay; when only the *rpsU2* 5' UTR is fused to *lacZ*, there is a ~4-fold increase in *lacZ* transcript abundance but β -galactosidase increases by only approximately 30%. This suggests that translation of the *rpsU2* operon mRNA, dependent on the *rpsU2* 5' UTR, is regulated by unknown mechanisms. 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.

To identify factors that limit *rpsU2* mRNA translation, we will take an unbiased pull-down approach. Specifically, we will use *in vitro* synthesized *rpsU2* 5' UTR with a 3' biotin moiety⁸⁵. This modified RNA, as well as a control modified RNA, will be incubated with cellular lysates from cells with and without bS21-2. After isolating the RNA-protein complexes using streptavidin beads, we will identify the co-purifying proteins by mass spectrometry and determine which proteins specifically interact with the *rpsU2* mRNA (but not a control mRNA). We will consider proteins that interact with the *rpsU2* mRNA in lysates with and without bS21-2 to be candidate regulators of *rpsU2* translation. To validate these candidates, we will then genetically modify *F. tularensis* cells to delete the encoding gene. We can then use qRT-PCR and reporter assays to assess the correlation between transcript abundance and translation. If the candidate does function to limit *rpsU2* translation, we expect to find that translation is increased in cells lacking bS21-2.

To maximize the possibility of finding regulators of bS21-2 production, we will take another, *in vivo*, approach. We have determined that cells containing the *rpsU2* 5' UTR is fused to *lacZ* have significantly more *lacZ* transcript but not substantially increased β -galactosidase activity (**Fig 4**). We will take advantage of this reporter and use transposon mutagenesis to screen for genes encoding products that inhibit translation of the *rpsU2* operon. Cells containing the *rpsU2* 5' UTR – *lacZ* reporter, either with or without bS21-2 have similar β -galactosidase activity. We will mutagenize cells lacking bS21-2 (which should have increased *lacZ* transcript abundance) and look for mutants with increased β -galactosidase activity. This screen should identify factors that allow increased translation of the *rpsU2* mRNA and potentially other factors that influence bS21-2 levels.

Aim 2 Potential pitfalls and alternate approaches

In Aim 2a, is that it is possible that bS21 homologs do not directly interact with the *rpsU2* 5' UTR to exert their effects or that they require an additional factor. If we do not identify evidence of direct binding between bS21-2 and the *rpsU2* 5' UTR via EMSA, we will identify additional factors using the *in vitro* pull-down approach described in Aim 2b above. Specifically, we will incubate the modified *rpsU2* 5' UTR with lysates from cells with and without bS21-2. After isolating and identifying the co-purifying proteins, we expect to observe one of two outcomes: (1) when incubated in lysate with bS21-2, the RNA associates with proteins likely to decrease mRNA stability or (2) when incubated in cellular lysates lacking bS21-2, the RNA associates with proteins likely to increase its stability. In both cases, we will consider candidate regulatory proteins those which specifically interact with the *rpsU2* 5' UTR in one condition but not the other and validate direct interaction using EMSAs. It is also possible that stability of the *rpsU2* 5' UTR is mediated by a small RNA, in which case we still expect to identify proteins specifically affecting RNA stability (e.g., Hfq, RNase E).

We propose to use EMSAs to evaluate interactions between bS21-2 and the *rpsU2* 5' UTR. An alternate approach is to use surface plasmon resonance (SPR) to evaluate direct binding and measure binding constants. The research groups have access to a Biacore T200 and could use this alternate approach.

In Aim 2b, we propose to identify and validate regulators of bS21 translation. If the identified regulators are essential, we will be unable to delete them to carry out the proposed validation studies. In this case, we will attempt to create mutants that are unable to bind RNA and/or use *in vitro* translation assays (as in Aim 1b) to validate translation repressors.

Aim 2 Timeline. We expect work on Aim 2a will begin in year 1 and extend through year 3. Aim 2b will initiate in year 3 and extend through year 5.

Aim 2 Summary

The ribosomal protein bS21-2 is important for intramacrophage survival of *F. tularensis*, but the factors that control its production have not been investigated. Here we show that production of bS21-2 is regulated at several steps post-transcriptionally. In particular, steady-state transcript abundance is influenced by all three bS21 homologs and translation of the bS21-2-encoding mRNA is controlled by unknown factor(s). In the work proposed in this aim, we will (1) identify the cis- and trans-acting factors that control abundance of the bS21-2-encoding transcript and (2) identify proteins that influence translation of this transcript. The experiments described will provide new insight and advances into our understanding of virulence gene regulation in *F. tularensis* as well as the mechanisms by which this human pathogen post-transcriptionally controls gene expression. In addition, these findings are expected to provide a broader insight into how cells with multiple homologs for non-zinc coordinating r-proteins regulate appropriate protein production.

Aim 3: Establish the effect of bS21 homologs on ribosome structure.

Rationale and Preliminary Results. We have demonstrated that *F. tularensis* bS21 homologs influence virulence gene expression, are not interchangeable, and that bS21-2 influences translation of specific mRNAs in *F. tularensis* (**Fig 2**)¹⁶. How bS21 homologs in γ -proteobacteria influence gene expression at the molecular level is unknown. We hypothesize that incorporation of different bS21 homologs influences translation initiation by altering the local ribosome structure.

As a preliminary study to determine if ribosomes containing different bS21 homologs have altered structure or conformation, we took a chemical biology approach. Antibiotics and antibiotic-resistance mutations have been used for many years as tools to monitor local structure in ribosome functional sites. Changes of residues in and around antibiotic binding sites can sufficiently perturb ribosome structure to influence antibiotic susceptibility. Because there are many translation inhibitors that function by binding to specific sites in the 30S subunit, we

used these to probe structure in specific areas of ribosomes^{86–88}. To assess cells containing ribosomes homogeneous for a single bS21 homolog, we created a set of isogenic strains encoding only one bS21-coding gene in the same genomic context. Specifically, these cells lack all three native genes encoding bS21 homologs and, at a neutral location (the Tn7 attachment site), encode a single bS21 homolog (**Table 1**)⁸⁴. We tested the sensitivity of these cells to various antibiotics to determine if a given bS21 homolog influences antibiotic efficacy.

Comparing the cells with different bS21 homologs, we found differential susceptibility to only a single antibiotic, kasugamycin (**Table 1**). In particular, cells containing only bS21-1 are significantly more resistant and cells containing only bS21-3 are significantly more sensitive to kasugamycin. Importantly, the limited differences in antibiotic susceptibility demonstrate that cells with different bS21 homologs have comparable cell permeability and 30S structure. Yet the altered susceptibility to kasugamycin is strong evidence that incorporation of distinct bS21 homologs leads to conformational changes in or around the kasugamycin binding site. Notably, kasugamycin specifically inhibits translation initiation and its binding site includes multiple rRNA nucleotides in close proximity to bS21^{89,90}. These results are consistent with incorporation of distinct bS21 homologs altering local ribosome structure and influencing translation initiation.

Available ribosome structures reveal that, in *E. coli* ribosomes, bS21 interacts directly with the 16S rRNA anti-Shine Dalgarno (ASD), a structure critical for translation initiation⁹¹. This raises the possibility that different bS21 homologs alter 16S rRNA structure around the mRNA channel to influence translation initiation. Additionally, given the location of bS21, the homologs could directly interact with 5' UTRs of mRNAs.

Different bS21 homologs may also influence a key post-transcriptional rRNA modification. Kasugamycin resistance can be conferred by the loss of KsgA (also known as RsmA), a universally conserved SAM-dependent dimethyltransferase^{92,93}. The function of KsgA is to dimethylate A1518 and A1519 (*E. coli* rRNA numbering) in helix 45 of the 16S rRNA^{94–96}. Cells lacking the dimethylation in helix 45 are kasugamycin-resistant due to changes in rRNA structure, in particular altered packing of 16S rRNA helices 45 and 44^{94–96}. Notably we have isolated kasugamycin-resistant *F. tularensis* (inhibitory zone diameter 16.1 mm +/- 0.36; compare to Table 1), which are *ksgA* mutants.

Post-transcriptional modifications made by KsgA occur during ribosome assembly, prior to formation of a fully active 30S particle^{94,96}. Although KsgA and bS21 do not directly bind to the same site, bS21 is inhibitory to KsgA activity and it has been suggested that bS21 stabilizes the 30S subunit in a conformation inaccessible to KsgA^{97,98}. It is possible that in *F. tularensis*, incorporation of different bS21 homologs into ribosomes permits different levels of 16S rRNA dimethylation, which subsequently influences translation initiation.

While we can use specific assays to quantitatively assess changes in local ribosome structure, a complementary approach is to solve the structure of *F. tularensis* ribosomes with each bS21 homolog. Although the ribosome is a large (~200 Å) macromolecular complex consisting of more than 50 proteins and three RNAs, advances in crystallography and cryogenic electron microscopy (cryo-EM) have led to many successful structural studies. Yet only few of these studies have focused on ribosomes from pathogenic bacteria, and no structural information is available for the *F. tularensis* ribosome^{99–104}. The Ramsey lab has successfully isolated pure *F. tularensis* 70S ribosomes from cells containing only a single bS21 homolog (from double deletion cells that lack two of three genes encoding bS21). The Schumacher laboratory specializes in the atomic-level understanding of protein-nucleic acid interactions, has significant expertise in cryo-EM structure determination, and has initiated solving the structure of the *F. tularensis* ribosome, currently resolved to better than 4 Å (**Fig 5**). The extremely high quality data obtained indicate that use of cryo-EM will be successful in solving the proposed ribosome structures.

To influence local ribosome structure and affect translation initiation, we hypothesize that bS21 could (1) alter the conformation of nearby 16S rRNA, (2) influence post-transcriptional modification of the 16S rRNA, and/or (3) bind directly to mRNAs. Because they are not mutually exclusive, we intend to specifically test each possibility and use a complementary unbiased approach to determine the

Table 1. Sensitivity of indicated *F. tularensis* cells to small ribosome subunit targeting antibiotics and ciprofloxacin

Antibiotic	Cells with All bS21 homologs	Only bS21-1	Only bS21-2	Only bS21-3
<i>kasugamycin</i>	33.5 +/- 1.4	30.5 +/- 0.4*	34.3 +/- 2.1	39.7 +/- 0.8*
<i>kanamycin</i>	53.5 +/- 0.6	55.3 +/- 0.2	53.6 +/- 0.4	53.2 +/- 0.5
<i>tetracycline</i>	46.0 +/- 0.9	45.8 +/- 1.4	49.0 +/- 1.9	47.2 +/- 0.3
<i>streptomycin</i>	55.1 +/- 3.4	54.9 +/- 0.7	56.1 +/- 0.1	58.0 +/- 1.8
<i>ciprofloxacin</i>	56.5 +/- 1.3	56.0 +/- 1.2	55.1 +/- 0.1	56.0 +/- 0.1

Values represent inhibitory zone diameters (mm) from disc diffusion assays. +/- values represent standard deviation; results are representative of at least two replicate experiments performed in biological triplicate

*Students t-test p < 0.05 compared to WT

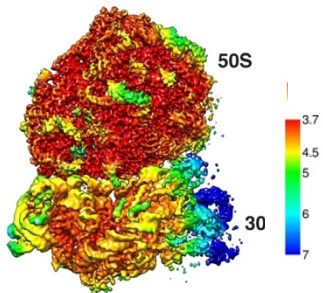


Figure 5. Density map of the *F. tularensis* ribosome. Rainbow scale corresponds to local resolution in Å.

effects of incorporating different bS21 homologs: solving the structures of each class of *F. tularensis* ribosomes. Together, the proposed studies will elucidate how incorporation of different bS21 homologs impacts ribosome structure and function, answering model-driven questions (Aim 3a) and taking an unbiased approach (Aim 3b). Our results will elucidate how conformational changes can regulate synthesis of proteins critical for virulence.

Research Design

Aim 3a. Establish how bS21 homologs alter local ribosome structure to impact translation initiation

We will use targeted approaches to determine how incorporation of bS21 homologs influence ribosome structure and translation initiation. We hypothesize that different bS21 homologs influence mRNAs-ribosome interactions by altering the rRNA conformation, differentially permitting a key rRNA modification, and/or through direct interactions with 5' UTRs to influence initiation complex formation on specific mRNAs.

To address these hypotheses, we will compare *F. tularensis* ribosomes containing different bS21 homologs and compare the region surrounding the mRNA exit channel. Specifically, we will compare dimethylation of helix 45 using primer extension, use chemical probing to evaluate the local 16S rRNA structure and bS21-mRNA interactions, and use electrophoretic mobility shift assays (EMSAs) to determine if bS21 homologs can bind directly to specific mRNAs.

To assess dimethylation of the 16S rRNA, we will use a robust and well-described method, primer extension analysis^{105,106}. This technique relies on reverse transcription reactions combining purified rRNA, a labeled DNA primer complementary to the 3' end of the 16S rRNA and reverse transcriptase. The reverse transcription reaction is inhibited by bulky modifications like methylation, so the polymerase will either be stopped by methylation of A1519/A1518 or a nearby conserved modification, methylation of G1516. The relative amounts of each product, visualized by gel electrophoresis, can be quantified to determine the relative amount of modification^{105,106}. In these analyses, we will use ribosomes from wild-type *F. tularensis*, *ksgA* mutants and ribosomes purified from cells containing only a single bS21 homolog (as in Table 1). We expect that products from reactions with wild-type 16S rRNA will have stopped at the first modified nucleotide (A1519), while products from reactions with 16S rRNA from *ksgA* mutants extend past A1519, due to lack of the dimethylation. If different bS21 homologs influence dimethylation, we expect to see varying amounts of halted and read-through products, which can be quantified by polyacrylamide gel electrophoresis. These analyses will reveal if different bS21 homologs influence KsgA-mediated 16S rRNA modification.

In order to determine if different bS21 homologs influence the conformation of surrounding 16S rRNA and/or mRNAs, we will use chemical footprinting^{107,108}. The bS21 r-protein binds to the 16S rRNA; exposure of ribosomes to hydroxy radicals will lead to cleavage of the rRNA at solvent-accessible nucleotides. SHAPE (selective 2'-hydroxyl acylation analyzed by primer extension) provides information regarding conformational flexibility¹⁰⁹. Other chemical probes, such as dimethylsulfate, provide base-specific information regarding solvent accessibility. The chemically-induced cleavages or modifications are detected using primer extension, as described above. By comparing the modifications among ribosomes with different bS21 content, we will identify which rRNA nucleotides, if any, have changed conformation (become more or less solvent-accessible) due to incorporation of different bS21 homologs. We will specifically analyze the 16S rRNA regions adjacent to the bS21 binding site in ribosomes with different bS21 homologs. To assess how bS21 homologs influence local mRNA structure, we can take the same approach. Specifically we will use ribosome with different bS21 content to form initiation complexes on specific mRNAs prior to chemical footprinting. This will allow us to compare how bS21 content impacts the mRNA solvent-accessibility and conformation, particularly around the mRNA channel. We will use mRNAs that we have demonstrated are regulated or not (**Fig 2**), including virulence gene mRNAs (such as *pdpA*) and others informed by Aim 1.

Finally, we will also determine if bS21 homologs, in the absence of ribosomes, can bind directly with mRNAs to influence translation initiation. To test direct binding, we will take advantage of electrophoresis mobility shift assays (EMSAs) as in Aim 2a, combining purified bS21 homologs and labeled mRNAs. We expect that if bS21 homologs directly bind to the 5' UTR of mRNAs, combining the two will result in a shift in mRNA mobility. As a positive control, we will also use mRNAs and ribosomes from wild-type cells. We will assess the potential for binding between each bS21 homolog and various regulated and non-regulated mRNAs as above. These experiments will be guided by our results from chemical footprinting assays, any structure determinations from Aim 3b, and any results from Aim 2a.

Aim 3b. Determine the atomic-resolution structure for *F. tularensis* ribosomes with each bS21 homolog

To take an unbiased approach to understanding how bS21 homologs influence ribosome conformation and translation, we will use cryo-EM to solve the structures of 70S ribosomes containing each bS21 homolog in complex with mRNA and initiating tRNAs. The Schumacher laboratory has already established a pipeline for

cryo-EM analyses of *F. tularensis* ribosomes, which includes a state-of-the-art FEI Titan Krios cryo-electron microscope (**Fig 5**). We will initiate these studies by examining the interaction of the *pdpA* mRNA with ribosomes containing bS21-2, as the presence of bS21-2-containing ribosomes has a significant impact on PdpA abundance¹⁶. Additional mRNAs, informed by results from Aim 1, will also be studied in complex with ribosomes homogenous for the other bS21 homologs. Finally, as ribosomes with altered bS21 content have altered sensitivity to the antibiotic kasugamycin, we will obtain structures of the relevant ribosomes and kasugamycin together. From the latter structures, we expect to deduce the molecular basis for the altered efficacy of kasugamycin in cells with varied bS21 content (**Table 1**). These studies may also provide further insight into both translation initiation and the mechanism of kasugamycin action.

We have initially focused on using cryo-EM to solve the structure of the *F. tularensis* 70S ribosome lacking bS21. Given the ability of bS21 to associate and disassociate with 70S ribosomes, we anticipated that bS21 may not be present without formation and stabilization of initiation complexes. In fact, our preliminary studies yielded an initial density map of the apo *F. tularensis* 70S ribosome that lacks density for bS21 despite the presence of bS21-2 in the purified ribosome sample (**Fig 5**). However, solving this structure will allow us to (1) optimize cryo-EM conditions and structure determination parameters, (2) provide key information about differences between the bS21 interaction site on *F. tularensis* ribosomes and other published ribosome structures, and (3) will provide an important point of reference to compare ribosomes containing bS21 homologs.

Future work will include using the information obtained from experiments in Aims 3a and 3b to validate the identification of key residues of bS21 necessary for bS21-mediated regulation using the reporter assays similar to those outlined in Aim 1.

Aim 3 Potential pitfalls and alternate approaches

In Aim 3a, we propose to use EMSAs to evaluate mRNA-bS21 interactions. An alternate approach is to use surface plasmon resonance (SPR) to evaluate direct binding and measure binding constants. The research groups have access to a Biacore T200 and could use this alternate approach.

As mentioned above, obtaining cryo-EM ribosome structures with bS21 homologs may require optimization. We anticipate that including mRNA and initiating tRNA will stabilize bS21 on the ribosome. If not, we will add excess bS21 prior to formation of the ribosome complex with tRNA/mRNA. For this purpose, we have recently succeeded in generating expression constructs for each of the *F. tularensis* bS21 homologs and have purified them to homogeneity. In addition, we can use crosslinking to stabilize the ribosome complexes prior to grid formation. Finally, if attempts to form active 70S translation complexes with bS21 are not successful, we will take similar approaches to prepare and solve the structure of bS21-containing 30S initiation complexes.

Aim 3 Timeline. We expect work on both Aim 3a and Aim 3b will begin in year 1 and extend through year 5.

Aim 3 Summary

While any one of three bS21 homologs can be incorporated into *F. tularensis* ribosomes, how they influence ribosome structure and activity is unknown. By probing cells with ribosomes containing different bS21 homologs using antibiotics, we have revealed changes in local conformation around the mRNA channel. In the work proposed here, we will use directed biochemical assays to assess how bS21 homologs influence ribosome structure locally and cryo-EM to solve entire structures of *F. tularensis* ribosomes with different bS21 homologs. Our structural studies of *F. tularensis* ribosomes are expected to reveal how bS21, and particular bS21 homologs, promote translation of specific mRNAs. These findings will be key to a detailed understanding of the molecular mechanism by which bS21 homologs in *F. tularensis* and other bacteria influence gene expression, which will open new and exciting avenues for further research. In particular, we expect to be able to predict (1) which residues of bS21 are key for regulating translation of mRNAs, (2) what determinants of mRNA allow regulation by bS21, and (3) how initiation of translation may be affected by incorporation of bS21. Our studies will represent the first structural studies of ribosomes containing different bS21 homologs. They are also expected to provide crucial information about the function of bS21 in ribosomes and gene regulation applicable to organisms across the bacterial phylogeny. Finally, these results will also provide rationale and preliminary data to investigate the development of ribosome-specific antibiotics.

Rigor / Reproducibility and Statistical Analysis. All genetic constructs will be verified by sequencing. We do not routinely passage strains. All datasets will be made available in public repositories. All cell-based experiments will be performed at least twice in biological triplicate. High-throughput sequencing and *in vitro* assays will be performed in triplicate. Experiments will be performed with technical replicates where appropriate. To assess significance, T-tests and ANOVA with parametric or non-parametric *post-hoc* testing will be performed, and data will be presented as means +/- standard deviation. Differences in the data will be considered significant when the probability value (p-value) or adjusted p-value is <5.0% (p-value or adj p-value < 0.05).

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