Examining how the small ribosomal protein bS21 regulates gene expression in pathogenic bacteria

1. Statement of problem

The aim of this study is to determine how a small ribosomal protein (r-protein), bS21, influences gene expression in two pathogenic bacteria. In the bacterial pathogen *Francisella tularensis*, the small r-protein bS21 provides a source of ribosome heterogeneity as the *F. tularensis* genome encodes 3 distinct bS21 paralogs. My preliminary work identified that loss of bS21-2 leads to a lower abundance of specific components of the *F. tularensis* type VI secretion system (T6SS), indicating that ribosomes containing bS21-2 may regulate expression of critical virulence genes. In *Staphylococcus aureus*, multiple isolates with increased resistance to the cell envelope-targeting antibiotics daptomycin and vancomycin, have mutations in the gene encoding bS21 (Basco et al., 2019; Friedman et al., 2006). This suggests that bS21 may regulate genes involved in antibiotic resistance in *S. aureus*. In the proposed work, we will identify genes regulated by bS21 in both *F. tularensis* and *S. aureus*, study the mechanism by which bS21 interacts (directly or indirectly) with mRNA, and investigate how these genes are regulated at the level of translation.

2. Justification of the study

2.1. Gene regulation at the level of translation

Research into the regulation of gene expression in bacteria has primarily focused on transcription, and has identified many clear mechanisms by which transcription controls gene expression. Translation is typically viewed as a constitutive event but significant work is now being done to investigate the role that translation may play in regulating gene expression, particularly under stress conditions. While transcription is undoubtedly a central point of regulation, the potential importance of gene regulation at the level of translation is exemplified in a study of yeast and *Escherichia coli* grown in minimal media (Lu et al., 2007). Using a novel protein abundance index, researchers found that only about half of *E. coli* protein levels are regulated at the level of transcription, consistent with significant regulation at the level

of translation and suggesting that control of translation may be key in stress conditions (Lu et al., 2007). There are a number of mechanisms by which gene expression may be regulated at the level of translation, including variations in recruitment of mRNA to the ribosome and factors that affect assembly of the translation initiation complex (Milón & Rodnina, 2012). Small RNAs (sRNAs) can also contribute to gene regulation post-transcriptionally in multiple ways, including binding to mRNAs to prevent or promote translation (Harris et al., 2013). Although it is clear that regulation of translation may influence gene expression, there are few studies that investigate how variations in composition of the translation machinery, the ribosome, influences gene expression.

2.2. Heterogeneous ribosomes may have specialized functions

The ribosome, which translates mRNA into protein, is highly conserved in all domains of life and consists of two subunits composed of ribosomal RNAs (rRNAs) and r-proteins. Typically, the ribosome is viewed as acting constitutively and without variability, translating every mRNA equally efficiently. In fact, our standard terminology reflects this, as we usually refer to *all* ribosomes when discussing their function (i.e. "Ribosomes contain the following proteins...") as opposed to distinguishing between different types of ribosomes (i.e. "*Most* ribosomes in *these* cells contain *this* protein homolog…"). Heterogeneity in ribosomes exists and arises via a number of mechanisms, including from diversity in rRNA sequences, r-proteins encoded by distinct homologs within the same cell, post-transcriptional modification of rRNA, and post-translational modification of r-proteins (Byrgazov et al., 2013; Sauret et al., 2015). The presence of these physical differences raises the possibility that heterogeneous ribosomes within the same organism have functional differences. The term "specialized ribosomes" describes structurally diverse ribosomes that may have altered activity (Xue & Barna, 2012).

In the context of eukaryotic organisms, there are a significant number of studies focused on specialized ribosomes (reviewed in Xue & Barna, 2012). While many studies in bacteria have reported heterogeneous ribosome populations, our understanding of any functional differences among these

heterogenous ribosomes remains limited (Kurland et al., 1969; Deusser, 1972). To continue to study specialized ribosomes in bacteria, it would be ideal to focus on systems in which ribosome heterogeneity is inherent and is limited or stems from a single source, which is the case for the systems we propose to study.

2.3. The role of bS21 in translation

It has been shown that the small r-protein bS21, a component of the 30S subunit, is important in initiation of translation. Specifically, mutants lacking bS21 or treated with anti-bS21 antibodies in *E. coli* are fully functional in fMet-tRNA binding but are unable to bind bacteriophage MS2 RNA (Van Duin & Wijnands, 1981). MS2 RNA has been commonly used in studying translation; the inability of ribosomes lacking bS21to bind MS2 RNA indicates disruption to translation initiation. These results suggest that bS21 is necessary to mediate the interaction between the ribosome and mRNA molecule (Van Duin & Wijnands, 1981). Additionally, ribosomes treated with 2-methoxy-5-nitrotropone, which have lost their ability to bind bacteriophage RNA due to chemical modification of r-protein lysines, regained this ability when four untreated purified r-proteins were added: bS1, uS12, uS13, and bS21 (Chang & Craven, 1977). This further suggests that bS21 is essential in moderating the specific interaction between 16S rRNA and mRNA. Crystallographic data reveals that in *E. coli*, bS21 is found close to the exit channel of RNA on

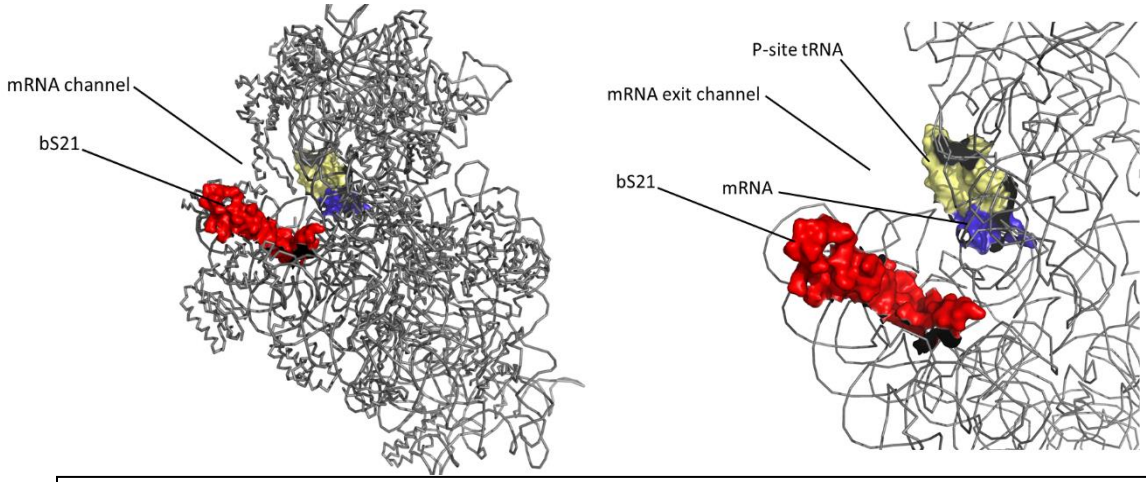


Figure 1. bS21 location in *E. coli* ribosome. Partial 30S subunit of *E. coli* ribosome by cryogenic electron microscopy (cryoEM) showing bS21 location relative to mRNA, at two magnifications. Red = bS21; blue = mRNA; grey = 16S rRNA and remaining 30S ribosomal proteins. PDB entry 4V50.

the small subunit (Berk et al., 2006; Figure 1). These structural results support the biochemical studies, as they identify bS21 in an ideal location to interact with the 5´ region of mRNA.

Despite the fact that bS21 seems to play an important role in translation initiation, many species lack the gene encoding bS21, *rpsU*, indicating that bS21 is not strictly essential for translation. Although non-essential, mutation or modification of bS21 in bacteria that encode *rpsU* results in specific phenotypes, suggesting that bS21 regulates specific processes. In *Bacillus subtilis*, bS21 mutants have defects in motility and biofilm formation (Takada et al., 2014) while null mutants in *Listeria monocytogenes* have increased acid stress resistance and changes in transcription of stress resistance genes (Metselaar et al., 2015; Metselaar et al., 2016). bS21 was identified as a virulence factor in *Burkholderia pseudomallei* during a transposon-sequencing (Tn-seq) mutagenesis screen in lung infections (Guttierez et al., 2015). Similarly, in a screen assessing the ability of *Francisella tularensis* transposon insertion mutants to replicate in a mouse infection model, the bS21-encoding gene *rpsU1* was identified as a virulence gene (Su et al., 2007). Finally, in *Staphylococcus aureus,* isolates with mutations in *rpsU* have increased resistance to daptomycin and vancomycin (Blake & O'Neill, 2013; Basco et al., 2019, Friedman et al., 2006). In each of these examples, no molecular mechanism has been proposed to explain the specific phenotypes in cells lacking bS21.

As one of the smallest and last-assembled r-proteins of the 30S subunit (Mizushima & Nomura, 1970), it is believed that bS21 is easily exchanged in *E. coli*. Notably, a bS21 homolog was found to be encoded by a cultivated pelagiphage, *Pelagibacter* phage HTVC008M, and the purified viral bS21 proteins were able to be incorporated into *E. coli* ribosomes (Mizuno et al., 2019). Additionally, using metagenomic analysis, over 1300 bS21 homologs were found in viral genomes, and these viruses infect a wide range of bacterial phyla, suggesting that horizontal exchange of bS21-encoding genes has occurred on numerous occasions (Mizuno et al., 2019). Furthermore, the high frequency with which bS21 genes are found on viral genomes raises the possibility that viruses may be utilizing bS21 to modify the

ribosomes of host cells, thereby improving the virus's ability to hijack the host machinery. The possibility of homolog switching, together with the literature on the structural and functional roles of bS21, leads us to hypothesize that bS21 is playing a role in the regulation of translation in specific processes.

2.4. *Francisella tularensis* encodes multiple bS21 homologs

While many organisms encode only one *rpsU* gene, most members of the *Francisella* genus, including the pathogenic bacteria *Francisella tularensis*, encode three homologs. *F. tularensis* is a Gram-negative, facultative intracellular bacteria that causes the disease tularemia (Sjöstedt, 2007). *F. tularensis* is remarkable in its ability to live in a diverse range of hosts, vectors, and environments. It can be found in ticks, flies, mosquitos, hares, and rodents, and can also survive in an aquatic environment (Sjöstedt, 2007). While capable of replicating in a wide variety of cell types, *F. tularensis* must be able to replicate in macrophage to cause disease (Nano et al., 2004). Because of this diversity of life styles, *F. tularensis* must regulate gene expression successfully to adapt to its many environments.

F. tularensis has a significantly reduced genome (Sjödin et al., 2012), yet retains three different copies of *rpsU*, which we refer to as *rpsU1*, *rpsU2*, and *rpsU3* and encode the bS21 proteins bS21-1, bS21-2, and bS21-3, respectively. At the amino acid level, the three homologs are highly variable, with percent identities ranging from 46-72% (Appendix 1, Supplemental Figure 1). Using amino acid sequences of 925 bS21 proteins from bacterial species of approximately 290 genera to build a phylogenetic tree, we determined that all three *F. tularensis* LVS bS21 proteins cluster together (Appendix 1, Supplemental Figure 2). This suggests that the homologs likely evolved from gene duplication events, leading us to categorize the genes as paralogs.

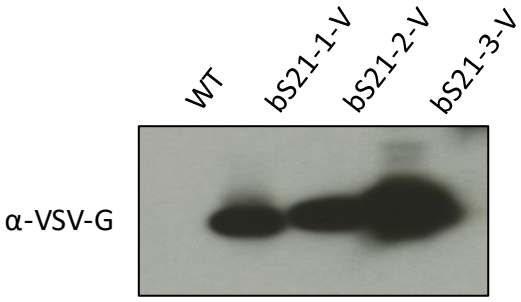


Figure 2. Incorporation of ectopically expressed bS21 in *F. tularensis* ribosomes. Immunoblot of purified ribosomes from wild-type (WT) cells and cells with ectopically expressed bS21 with VSV-G tags.

My preliminary research determined that at least bS21-2 and bS21-3 are incorporated into ribosomes in

wild-type *F. tularensis* cells grown in our standard laboratory conditions, and all three proteins can become incorporated into ribosomes after ectopic expression from a plasmid (Figure 2). These data suggest that ribosomes in *F. tularensis* are heterogenous with respect to bS21. Given that there are no other r-proteins with multiple homologs and all three copies of the rRNA genes are identical in *F. tularensis*, bS21 is the only apparent source of ribosome heterogeneity. This makes *F. tularensis* an ideal system in which to study the influence of ribosomes containing different bS21 paralogs on gene expression. Moreover, I have found that loss of *F. tularensis rpsU2* results in an intramacrophage growth defect (Appendix 1, Supplemental Figure 5). This finding suggests that bS21 paralogs may play a role in virulence, a topic which will be further explored throughout this study.

2.4.1. The F. tularensis *type VI secretion system is critical for virulence*

We have found that loss of bS21-2 in *F. tularensis* leads to defects in intramacrophage growth. This may be, at least in part, because cells lacking bS21-2 (Δ*rpsU2*), have reduced abundance of specific type VI secretion system (T6SS) proteins. The T6SS in *F. tularensis*, considered a subtype 2 T6SS (Russell et al., 2014), is absolutely necessary for virulence and intramacrophage growth (Nano et al., 2004). The T6SS is encoded on the *Francisella* pathogenicity island (FPI), which is duplicated in the *F. tularensis* genome (Nano & Schmerk, 2007). Each FPI encodes two convergent operons encoded on opposite strands: one with four genes (*iglA, iglB, iglC,* and *iglD*) and the other containing twelve genes, including *pdpA, pdpB, iglE,* and others (Appendix 1, Supplemental Figure 3). The FPI is known to be controlled at the level of transcription by the coordinate action of several factors, including MglA, SspA, and PigR (Brotcke et al., 2006;

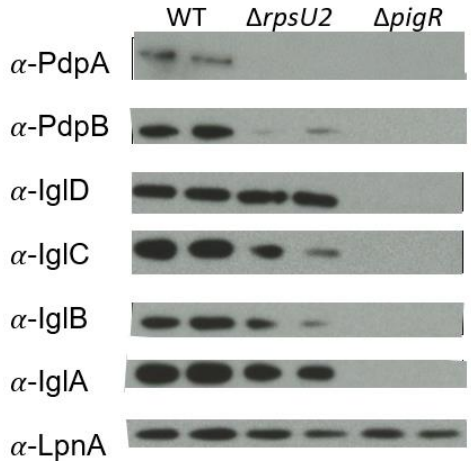


Figure 3. Differential expression of T6SS proteins in cells lacking *rpsU2* and *pigR*. Immunoblots of whole cell lysates from wild-type *F. tularensis* and indicated derivatives in duplicate, with specified antibodies. LpnA serves as a loading control.

Brotcke & Monack, 2008; Charity et al., 2007; Ramsey et al., 2015; Rohlfing et al., 2018; Cuthbert et al., 2017).

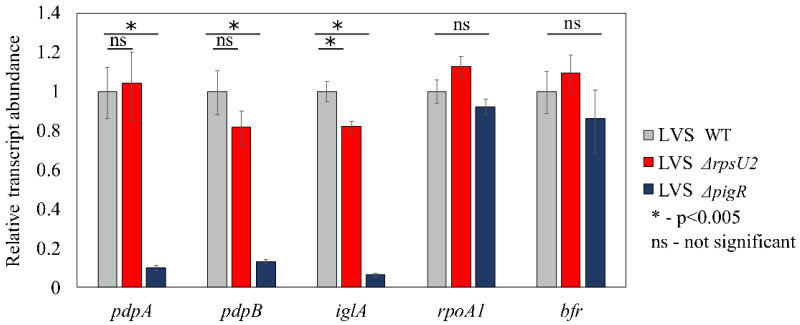


Figure 4. Differences in transcript abundance of *F. tularensis* cells lacking *rpsU2* and *pigR*. qRT-PCR analysis of cDNA from biological triplicates, performed in technical duplicate and normalized to *tul4* (which encodes LpnA) transcript abundance. Transcripts assessed on on x-axis, with *rpoA1* and *bfr* serving as controls

Using immunoblots with antibodies specific to FPI proteins, I found that loss of bS21-2 (LVS Δ*rpsU2)* results in major reductions in abundance of PdpA and PdpB, both of which are encoded on the same FPI operon (Figure 3). The proteins encoded on the *iglA-D* operon, however, remain relatively unchanged. I have also found that transcript abundance of *pdpA* and *pdpB* are essentially the same between wild-type cells and cells lacking bS21-2 (Figure 4), indicating that the changes in protein abundance are not due to reductions in transcript abundance. Cells lacking PigR (LVS Δ*pigR*) are used as a control for these experiments, because PigR regulates protein abundance of all FPI genes at the level of transcription. My results are consistent with a model in which the protein encoded by *rpsU2*, bS21-2, promotes translation of the virulence gene transcripts *pdpA* and *pdpB* in *F. tularensis*. The proposed study will further explore this model to examine the mechanism by which bS21-2 influences abundance of specific proteins to regulate gene expression.

2.5. Antibiotic-resistance is an increasing problem in *S. aureus*

Staphylococcus aureus is a Gram-positive facultative human pathogen frequently found on the skin and mucosal membranes of humans (Grace & Fetsch, 2018). *S. aureus* has been the subject of much research because of the relatively high rates of infection globally and the remarkable ability of *S. aureus* strains to become antibiotic-resistant (Chambers & Deleo, 2009). In fact, the attempt to treat *Staphylococcus* infections has led to the discovery or clinical adaptation of a number of antibiotics, including penicillin, methicillin and vancomycin (Chambers & Deleo, 2009). Each of these antibiotics has

eventually become insufficient in the treatment of *S. aureus* as resistant strains continually emerge, including methicillin-resistant *S. aureus* (MRSA) and vancomycin-resistant *S. aureus* (VRSA; Chambers & Deleo, 2009). Two of the more common drugs used to treat MRSA, daptomycin and vancomycin, both target synthesis of the cell envelope (Williams & Bardsley, 1999; Müller et al., 2016). Because *S. aureus* continues to gain antibiotic resistance regardless of the antibiotic used, it is critical to understand how antibiotic resistance is acquired and regulated so new therapeutics can be developed.

2.5.1. Vancomycin and daptomycin are important S. aureus *antibiotics*

Although vancomycin is the preferred treatment option for most *S. aureus* infections, the high concentrations necessary for effective treatment can have negative side effects (Holland et al., 2014). Vancomycin prevents bacterial survival by blocking synthesis of cell wall peptidoglycan, specifically by forming hydrogen bonds with D-alanine-D-alanine peptides (Williams & Bardsley, 1999). While daptomycin is an alternative antibiotic for treatment of VRSA strains, its mechanism of action is incompletely understood. However, its bactericidal effects on Gram-positive organisms may be attributed to its ability to bind the cell membrane and obstruct its electrical potential (*Daptomycin for methicillin…*). Clinical resistance to vancomycin and daptomycin is well documented and there are a number of genetic changes associated with increased resistance (Friedman et al., 2006). Among those changes includes loss-of-function mutations in *rpsU* (Blake & O'Neill, 2013; Basco et al., 2019; Friedman et al., 2006).

2.5.2. S. aureus rpsU *influences antibiotic susceptibility*

In *S. aureus,* mutations in its single *rpsU* gene have been identified in isolates with increased resistance to daptomycin and vancomycin (Basco et al., 2019; Friedman et al., 2006). Additionally, transposon library screening has confirmed that the *rpsU* gene is non-essential and its disruption is sufficient to alter resistance to both daptomycin and vancomycin (Blake & O'Neill, 2013). Increased cell wall thickness is correlated with increased antibiotic resistance (Howden et al., 2010), so the fact that mutations in *rpsU* lead to changes in antibiotic resistance for two antibiotics that target the cell envelope

suggests that bS21 may play a role in regulating cell envelope thickness (Silhavy et al., 2010). Specifically, we propose a model in which ribosomes containing bS21 differentially translate proteins involved in cell envelope regulation. The proposed study focuses on understanding the effect of bS21 in *S. aureus* using genetic manipulations, antibiotic susceptibility testing, and assessments of cell envelopes. The investigation of how *rpsU* influences antibiotic resistance in *S. aureus* provides another excellent model system to utilize in our investigation of the role of bS21 in gene regulation.

3. Experimental Methods

3.1. Aim 1: How does bS21-2 regulate gene expression in *Francisella tularensis?*

3.1.1. Rationale

Because of its physical location in the ribosome (Berk et al., 2006) and demonstrated role in translation initiation (Van Duin & Wijnands, 1981), we hypothesize that bS21 is involved in promoting translation of specific transcripts at the stage of translation initiation by interacting with mRNA leader sequences. Distinct amino acid residues in the three *F. tularensis* bS21 paralogs may preferentially bind to specific sequences or structural motifs in the 5´ untranslated region (UTR) of mRNA, leading to higher affinity interactions and increased translation of distinct species of mRNA.

3.1.2. Testing the contribution of mRNA 5´ UTRs

We will test the above hypothesis using *lacZ* reporter assays. Specifically, we will generate a vector that fuses a strong promoter, P_{bfr}, to the 5´ UTR of *pdpA*, followed by *lacZ*, which encodes β-galactosidase (P_{bfr}-UTR^{pdpA}-*lacZ*). We have demonstrated that transcription of *bfr* is not affected by loss of *rpsU2* at the level of transcription (Figure 4) so we expect that the *bfr* promoter will allow for the initiation of comparable amounts of transcription in wild-type and Δ*rpsU2* cells. We chose the *pdpA* 5´ UTR because we know *pdpA* is regulated by bS21-2 (Figure 3) and has a defined 5´ UTR (Ramsey et al., 2015). We will design the vector to integrate into a neutral location in the *F. tularensis* genome using a Tn7 transposon (Lovullo et al., 2009). We will integrate the vector into both the wild-type strain and the

Δ*rpsU2* strain. We will also generate a vector using the same promoter and *lacZ* gene, but it will contain the 5´ UTR of *bfr* (P_{bfr}-UTR^{bfr}-*lacZ*), which will serve as a control because abundance of the protein encoded by *bfr*, bacterioferritin, is not regulated by bS21-2 (Appendix 1, Supplemental Figure 4). We will normalize the amount of β-galactosidase produced in cells with the *pdpA* reporter to the amount produced in cells with the *bfr* reporter. Based on our hypothesis, we expect a higher relative amount of β-galactosidase in wild-type cells with our *pdpA* 5´ UTR reporter compared to cells lacking *rpsU2*, because we predict that ribosomes lacking bS21-2 will not efficiently interact with the 5´ UTR of *pdpA*.

We will perform β-galactosidase assays as essentially described by Dove & Hochschild (2004), in which we grow cells containing the appropriate constructs, permeabilize the cells, add a substrate for β-galactosidase, and quantify activity of β-galactosidase (see Appendix 2 for detailed methods). If our hypothesis is confirmed by this preliminary experiment, we will further investigate what characteristics of the *pdpA* 5´ UTR allow for increased translation by ribosomes containing bS21-2. Using secondary structure prediction software (mfold, University of Albany), we have determined that the 23 nucleotides of the *pdpA* 5´ UTR may form a stem-loop. We will mutate nucleotides to disrupt this structure (Appendix 1, Supplemental Table 1) in a new reporter vector, such that we can test the effect of a modified 5´ UTR on β-galactosidase production. We hypothesize that without the stem-loop structure, we will see no difference in β-galactosidase expression between cells with and without bS21-2. Additionally, we can determine if this is structure-specific by making complementary mutations (Appendix 1, Supplemental Table 1) to restore the stem-loop structure. If we see a return to the original results of the β-galactosidase assays, it will strongly suggest that the stem-loop structure is key for the recognition of the 5´ UTR by bS21-2.

If we do not observe significantly more β-galactosidase activity in wild-type cells compared to those lacking bS21-2, there are several possibilities to consider. First, RNA-Seq data (Ramsey & Dove, 2016) suggests *pdpA* may have an upstream, alternate transcription start site. If this is the case, bS21-2

may only recognize this longer 5´ UTR, so we can attempt the same experiment described above with a construct containing the longer 5´ UTR. If we still see no significant difference in β-galactosidase expression in wild-type versus cells lacking *rpsU2* in the described reporter constructs, it would suggest that there is another mechanism by which bS21-2 is regulating the abundance of PdpA and PdpB. This regulation could be indirect, such as if bS21-2 influences translation of a protease, and this will require further investigation. Specifically, we will employ a proteomics approach by using mass spectrometry on whole cell lysates of wild-type cells and cells lacking bS21-2. Using this approach, we could identify other proteins with significant differences in abundance that might be attributed to the presence or absence of bS21-2. A protein that is more abundant in wild-type cells than cells lacking bS21-2 may be interacting with bS21-2 to moderate the protein-level changes we see in PdpA and PdpB.

3.1.3. In vitro *translation assays*

If our reporter assays reveal that the 5´ UTR is essential for the regulation of PdpA protein abundance, it is possible either that there is a direct interaction between the 5´ UTR and bS21-2 or that another protein (or proteins) mediates the observed regulation. To determine if ribosomes containing bS21-2 are sufficient to regulate *pdpA* mRNA translation, we will utilize the PURExpress Δ Ribosome Kit from NEB. The PURExpress Δ Ribosome Kit contains tRNAs, initiation factors, elongation factors, and all other components necessary for translation. This kit lacks ribosomes, so we will be able to perform this experiment with *F. tularensis* ribosomes with and without bS21-2.

First, we will isolate ribosomes using a protocol adapted from Moazed & Noller (1989), in which successive sucrose cushions are used to purify 70S ribosomes from lysed cells (Appendix 2). We will purify ribosomes from strains that contain only one of the bS21 homologs, so we will isolate ribosomes from cells lacking *rpsU1* and *rpsU3* (which contain only bS21-2), cells lacking *rpsU1* and *rpsU2* (which contain only bS21-3), and cells lacking *rpsU2* and *rpsU3* (which contain only bS21-1). We will then use

these in the PURExpress® Δ Ribosome Kit, along with a modified version of the provided template plasmid with the T7 promoter and our genes of interest. We will compare translation of *pdpA* to a control gene unaffected bS21-2, *bfr*, in a single reaction, expressing both proteins in one reaction. This approach, which has been successfully utilized in other models, will control for experimental variability in transcription-translation efficiency (Ashara & Chong, 2010). We will analyze the resulting protein abundances using immunoblotting with antibodies specific to each protein. We expect to see more PdpA produced in reactions with ribosome containing bS21-2 than with the ribosomes containing other bS21 paralogs. If we do not find a difference in PdpA protein abundance between different ribosome populations, it is possible that there is another protein moderating the interaction between the 5´ UTR and bS21-2. To attempt to identify this protein, we can use two approaches: a biochemical approach and a genetic approach. For the biochemical method, we can use anti-VSV-G beads to purify VSV-G-tagged bS21-2 and associated proteins. Specifically, we would use cells lacking the endogenous bS21-2 but ectopically producing VSV-G tagged bS21-2 (Δ*rpsU2* pF-*rpsU2*-V). We will then use mass spectrometry to identify which proteins co-purify with bS21-2, as one of these proteins may be moderating the observed interaction with the *pdpA* transcript. For the genetic method, we will perform a transposon mutagenesis experiment on wild-type cells containing a reporter, specifically the *bfr* promoter and 5´ UTR of *pdpA* fused to lacZ (P_{bfr}-UTR^{pdpA}-*lacZ;* previously constructed). Colonies that have less β-galactosidase production (less blue when plated on X-gal), will be isolated; we expect that the gene(s) disrupted in these cells will encode proteins necessary for the efficient translation of *pdpA*. Together, these experiments should provide significant insights into the mechanism that bS21-2 uses to regulate gene expression at the level of translation in *F. tularensis.*

3.2. Aim 2: Does bS21 play a role in daptomycin- and vancomycin-resistance of *Staphylococcus aureus* through alterations in cell envelope structure?

3.2.1. Rationale

Increased resistance to daptomycin and vancomycin, two cell envelope-targeting antibiotics, is associated with mutations in the single *rpsU* gene of *S. aureus* clinically (Basco et al., 2019, Friedman et al., 2006). The association appears to be direct, as transposon-mediated inactivation of *rpsU* also leads to higher resistance to daptomycin and vancomycin (Blake & O'Neill, 2013). Although the mechanism by which loss of *rpsU* leads to increased vancomycin and daptomycin resistance is unknown, one way *S. aureus* can become more resistant to vancomycin is to increase cell wall thickness (Cui et al., 2006). This leads us to hypothesize that loss of bS21 might result in increased cell wall thickness and lead to increased resistance to daptomycin and vancomycin. Specifically, we propose that bS21 may regulate production of cell envelope proteins, thereby affecting cell envelope size and antibiotic resistance. The following experiments will attempt to answer this, by deleting *rpsU* in *S. aureus*, confirming alterations in antibiotic resistance, and determining how *rpsU* may influence cell-envelope thickness.

3.3.2. Deletion of rpsU

We will first confirm the previously observed phenotype of vancomycin- and daptomycin-resistance by creating a clean deletion of *rpsU* in *S. aureus.* To do so, we will follow a protocol outlined in Kato & Sugai (2011) and explained in depth in Appendix 2. In short, we will use an allelic replacement strategy. The vector we use will contain two regions flanking *rpsU* with the gene itself missing, genes encoding ampicillin and tetracycline resistances, a constitutive origin for plasmid replication in *E. coli*, and a temperature-sensitive origin for plasmid replication in *S. aureus*. The plasmid will be created and propagated in *E. coli* then passed through *S. aureus* RN4220, a strain that will modify the plasmid to protect it from nucleases in our target strain, *S. aureus* HG003. After transformation into *S. aureus* HG003, we will confirm integration of the vector using colony PCR and then screen for cells that have crossed out *rpsU* and the remainder of the plasmid and are thus tetracycline-sensitive.

3.3.3. Population analysis of S. aureus *antibiotic resistance*

In order to confirm reports of changes in vancomycin- and daptomycin-resistance due to loss of bS21, we will use the Δ*rpsU* strain to test susceptibility of the bacteria to each of these antibiotics. Using a protocol adapted from Hanaki & Hiramatsu (2001) with more details in Appendix 2, we will first grow our wild-type and Δ*rpsU* strains in culture overnight and normalize the optical density of cell cultures to A_{578}=0.3 to assure all plates start with the same number of colony-forming units (CFUs, approximately 10^8). We will create plates containing serial dilutions of each antibiotic, with daptomycin ranging from 0.039 – 5 μg/mL and vancomycin ranging from 0.078 – 10 μg/mL, based on published minimum inhibitory concentrations for *S. aureus (*Sader et al., 2010). We will spread serial dilutions of the cells onto plates of each antibiotic concentration in triplicate and incubate the plates for 48 hours. We will then determine the MIC as the lowest concentration of antibiotic that resulted in no colonies. We expect, based on previous studies, that Δ*rpsU* cells will have a higher MIC for both daptomycin and vancomycin when compared to wild-type *S. aureus.*

3.3.4. Analysis of cell envelope size

Previous research has shown that *S. aureus* cells with increased resistance to vancomycin (vancomycin-intermediate *S. aureus,* or VISA) have thickened cell envelopes (Cui et al., 2006). In order to test whether loss of bS21 also results in a thickened cell envelope, we will use transmission electron microscopy (TEM) and prepare samples using a protocol adapted from Hanaki et al. (1998). Details of the sample preparation can be found in Appendix 2. In short, we will fix mid-log cells in glutaraldehyde, stain with osmium tetroxide, then embed in resin, prepare thin sections, and stain again. We will then analyze using TEM at x30,000 to measure the cell envelope thickness. We expect to find a thickened cell envelope in cells lacking *rpsU* compared to wild-type cells and hypothesize that this will correlate with increased vancomycin- and daptomycin-resistance. If we do not find that the cell envelopes are thicker in Δ*rpsU* cells, there are two approaches we can use to investigate the connection between bS21 and resistance.

Specifically, we could use a proteomics approach similar to described for *F. tularensis*, in which we compare whole cell lysates of wild-type and Δ*rpsU* cells via mass spectrometry to identify proteins that differ in abundance when *rpsU* is lost. Additionally, we could use RNA-Seq to compare transcript abundance genome-wide in wild-type cells to Δ*rpsU* cells because changes in transcription may reflect changes in protein abundance.

4. Resources required

The majority of resources required for these sets of experiments will be provided by Kathryn Ramsey's lab. Sequencing will be completed at the Rhode Island Genomics and Sequencing Center. Microscopy will be completed using resources from the Rhode Island Consortium for Nanoscience and Nanotechnology. Immunoblotting analyses will be completed at the Rhode Island INBRE core facility.

References

Asahara, H., & Chong, S. (2010). *In vitro* genetic reconstruction of bacterial transcription initiation by coupled synthesis and detection of RNA polymerase holoenzyme. *Nucleic Acids Research, 38*(13), e141. doi:10.1093/nar/gkq377

Basco, M. D. S., Kothari, A., Mckinzie, P. B., Revollo, J. R., Agnihothram, S., Azevedo, M. P., Saccente, M., Hart, M. E. (2019). Reduced vancomycin susceptibility and increased macrophage survival in *Staphylococcus aureus* strains sequentially isolated from a bacteraemic patient during a short course of antibiotic therapy. *Journal of Medical Microbiology, 68*(6), 848. doi:10.1099/jmm.0.000988

Berk, V., Zhang, W., Pai, R. D., Cate, J. H. D., & Cate, J. H. D. (2006). Structural basis for mRNA and tRNA positioning on the ribosome. *Proceedings of the National Academy of Sciences of the United States of America, 103*(43), 15830. doi:10.1073/pnas.0607541103

Blake, K. L., & O'Neill, A. J. (2013). Transposon library screening for identification of genetic loci participating in intrinsic susceptibility and acquired resistance to anti-staphylococcal agents. *Journal of Antimicrobial Chemotherapy, 68*(1), 12-16. doi:10.1093/jac/dks373

Brotcke, A., & Monack, D. M. (2008). Identification of *fevR*, a novel regulator of virulence gene expression in *Francisella novicida*. *Infection and Immunity, 76*(8), 3473. doi:10.1128/IAI.00430-08

Brotcke, A., Weiss, D. S., Kim, C. C., Chain, P., Malfatti, S., Garcia, E., & Monack, D. M. (2006). Identification of MglA-regulated genes reveals novel virulence factors in *Francisella tularensis*. *Infection and Immunity, 74*(12), 6642. doi:10.1128/IAI.01250-06

Byrgazov, K., Vesper, O., & Moll, I. (2013). Ribosome heterogeneity: Another level of complexity in bacterial translation regulation. *Current Opinion in Microbiology, 16*(2), 133-139. doi:10.1016/j.mib.2013.01.009

Chambers, H. F., & Deleo, F. R. (2009). Waves of resistance: *Staphylococcus aureus* in the antibiotic era. *Nature Reviews.Microbiology, 7*(9), 629. doi:10.1038/nrmicro2200

Chang, C., & Craven, G. R. (1977). Identification of several proteins involved in the messenger RNA binding site of the 30 S ribosome by inactivation with 2-methoxy-5-nitrotropone. *Journal of Molecular Biology, 117*(2), 401-418. doi:10.1016/0022-2836(77)90135-8

Charity, J. C., Costante-Hamm, M., Balon, E. L., Boyd, D. H., Rubin, E. J., & Dove, S. L. (2007). Twin RNA Polymerase–Associated proteins control virulence gene expression in *Francisella tularensis*. *PLoS Pathogens, 3*(6), e84. doi:10.1371/journal.ppat.0030084

Cui, L., Iwamoto, A., Lian, J., Neoh, H., Maruyama, T., Horikawa, Y., & Hiramatsu, K. (2006). Novel mechanism of antibiotic resistance originating in vancomycin-intermediate *Staphylococcus aureus*. *Antimicrobial Agents and Chemotherapy, 50*(2), 428. doi:10.1128/AAC.50.2.428-438.2006

Cuthbert, B. J., Ross, W., Rohlfing, A. E., Dove, S. L., Gourse, R. L., Brennan, R. G., & Schumacher, M. A. (2017). Dissection of the molecular circuitry controlling virulence in *Francisella tularensis*. *Genes & Development, 31*(15), 1549. doi:10.1101/gad.303701.117

Daptomycin for Methicillin-Resistant and Methicillin-Sensitive Staphylococcus Aureus Infection: A Review of Clinical Effectiveness, Cost-Effectiveness and Guidelines [Internet]. Ottawa (ON): Canadian Agency for Drugs and Technologies in Health; 2016 Jan 21. Available from: https://www.ncbi.nlm.nih.gov/books/NBK343998/

Deusser, E. (1972). Heterogeneity of ribosomal populations in *Escherichia coli* cells grown in different media. *Molecular & General Genetics, 119*(3), 249-258. doi:10.1007/BF00333862

Dove, S. L., & Hochschild, A. (2004). A bacterial two-hybrid system based on transcription activation. In H. Fu (Ed.), *Protein-protein interactions: Methods and applications* (pp. 231-246). Totowa, NJ: Humana Press. doi:10.1385/1-59259-762-9:231 Retrieved from https://doi.org/10.1385/1-59259-762-9:231

Friedman, L., Alder, J. D., & Silverman, J. A. (2006). Genetic changes that correlate with reduced susceptibility to daptomycin in *Staphylococcus aureus*. *Antimicrobial Agents and Chemotherapy, 50*(6), 2137. doi:10.1128/AAC.00039-06

Grace, D., & Fetsch, A. (2018). In Fetsch A. (Ed.), *Chapter 1* - Staphylococcus aureus—*A foodborne pathogen: Epidemiology, detection, characterization, prevention, and control: An overview* Academic Press. doi:https://doi-org.uri.idm.oclc.org/10.1016/B978-0-12-809671-0.00001-2

Gutierrez, M. G., Yoder-Himes, D. R., & Warawa, J. M. (2015). Comprehensive identification of virulence factors required for respiratory melioidosis using Tn-seq mutagenesis. *Frontiers in Cellular and Infection Microbiology, 5*, 78. doi:10.3389/fcimb.2015.00078

Hanaki, H., Kuwahara-Arai, K., Boyle-Vavra, S., Daum, R. S., Labischinski, H., & Hiramatsu, K. (1998). Activated cell-wall synthesis is associated with vancomycin resistance in methicillin-resistant *Staphylococcus aureus* clinical strains Mu3 and Mu50. *The Journal of antimicrobial chemotherapy, 42*(2), 199–209. https://doi-org.uri.idm.oclc.org/10.1093/jac/42.2.199

Hanaki, H., & Hiramatsu, K. (2001). Detection Methods of Glycopeptide-Resistant *Staphylococcus aureus* I: Susceptibility Testing. *Methods in molecular medicine, 48*, 85–91. https://doi-org.uri.idm.oclc.org/10.1385/1-59259-077-2:85

Harris, J. F., Micheva-Viteva, S., Li, N., & Hong-Geller, E. (2013). Small RNA-mediated regulation of host- pathogen interactions. *Virulence, 4*(8), 785–795. https://doi.org/10.4161/viru.26119

Holland, T. L., Arnold, C., & Fowler, V. G. (2014). Clinical management of *Staphylococcus aureus* bacteremia: A review. *JAMA, 312*(13), 1330. doi:10.1001/jama.2014.9743

Howden, B. P., Davies, J. K., Paul, D. R. J., Stinear, T. P., & Lindsay Grayson, M. (2010). Reduced vancomycin susceptibility in *Staphylococcus aureus*, including vancomycin-intermediate and heterogeneous vancomycin-intermediate strains: Resistance mechanisms, laboratory detection, and clinical implications. *Clinical Microbiology Reviews, 23*(1), 99-139. doi:10.1128/CMR.00042-09

Kato, F., & Sugai, M. (2011). A simple method of markerless gene deletion in *Staphylococcus aureus*. *Journal of Microbiological Methods, 87*(1), 76-81. doi:10.1016/j.mimet.2011.07.010

Kurland, C. G., Voynow, P., Hardy, S. J. S., Randall, L., & Lutter, L. (1969). Physical and functional heterogeneity of *E. coli* ribosomes. *Cold Spring Harbor Symposia on Quantitative Biology, 34*, 17-24. doi:10.1101/sqb.1969.034.01.006

Lovullo, E. D., Molins-Schneekloth, C., Schweizer, H. P., & Pavelka, M. S. (2009). Single-copy chromosomal integration systems for *Francisella tularensis*. *Microbiology, 155*(4), 1152. doi:10.1099/mic.0.022491-0

Lu, P., Vogel, C., Wang, R., Yao, X., & Marcotte, E. M. (2007). Absolute protein expression profiling estimates the relative contributions of transcriptional and translational regulation. *Nature biotechnology*, *25*(1), 117–124. https://doi.org/10.1038/nbt1270

Metselaar, K. I., den Besten, H. M., Boekhorst, J., van Hijum, S. A., Zwietering, M. H., & Abee, T. (2015). Diversity of acid stress resistant variants of *Listeria monocytogenes* and the potential role of ribosomal protein S21 encoded by *rpsU*. *Frontiers in microbiology*, *6*, 422. https://doi.org/10.3389/fmicb.2015.00422

Metselaar, K. I., Abee, T., Zwietering, M. H., & Den Besten, H., M.W. (2016). Modeling and validation of the ecological behavior of wild-type *Listeria monocytogenes* and stress-resistant variants. *Applied and Environmental Microbiology, 82*(17), 5389. doi:10.1128/AEM.00442-16

Milón, P., & Rodnina, M. V. (2012). Kinetic control of translation initiation in bacteria. *Critical reviews in biochemistry and molecular biology*, *47*(4), 334–348. https://doi.org/10.3109/10409238.2012.678284

Mizuno, C. M., Guyomar, C., Roux, S., Régis Lavigne, Francisco Rodriguez-Valera, Sullivan, M. B., . . . Krupovic, M. (2019). Numerous cultivated and uncultivated viruses encode ribosomal proteins. *Nature Communications, 10*(1), 1-11. doi:10.1038/s41467-019-08672-6

Mizushima, S., & Nomura, M. (1970). Assembly mapping of 30S ribosomal proteins from *E. coli*. *Nature, 226*(5252), 1214. doi:10.1038/2261214a0

Moazed, D., & Noller, H. F. (1989). Interaction of tRNA with 23S rRNA in the ribosomal A, P, and E sites. *Cell, 57*(4), 585-597. doi:10.1016/0092-8674(89)90128-1

Müller, A., Wenzel, M., Strahl, H., Grein, F., Terrens, N. V. S., Kohl, B., . . . Hamoen, L. W. (2016). Daptomycin inhibits cell envelope synthesis by interfering with fluid membrane microdomains. *Proceedings of the National Academy of Sciences - PNAS, 113*(45), E7077-E7086. doi:10.1073/pnas.1611173113

Nano, F. E., & Schmerk, C. (2007). The *Francisella* pathogenicity island. *Annals of the New York Academy of Sciences, 1105*, 122.

Nano, F. E., Zhang, N., Cowley, S. C., Klose, K. E., Cheung, K. K. M., Roberts, M. J., . . . Elkins, K. L. (2004). A *Francisella tularensis* pathogenicity island required for intramacrophage growth. *The Journal of Bacteriology, 186*(19), 6430. doi:10.1128/JB.186.19.6430-6436.2004

Ramsey, K. M., & Dove, S. L. (2016). A response regulator promotes *Francisella tularensis* intramacrophage growth by repressing an anti-virulence factor. *Molecular Microbiology, 101*(4), 688-700. doi:10.1111/mmi.13418

Ramsey, K. M., Osborne, M. L., Vvedenskaya, I. O., Su, C., Nickels, B. E., & Dove, S. L. (2015). Ubiquitous promoter-localization of essential virulence regulators in *Francisella tularensis*. *PLoS Pathogens, 11*(4), e1004793. doi:10.1371/journal.ppat.1004793

Rohlfing, A. E., Ramsey, K. M., & Dove, S. L. (2018). Polyphosphate kinase antagonizes virulence gene expression in *Francisella tularensis*. *Journal of Bacteriology, 200*(3) doi:10.1128/JB.00460-17

Russell, A., Wexler, A., Harding, B., Whitney, J., Bohn, A., Goo, Y., . . . Chou, S. (2014). A type VI secretion-related pathway in bacteroidetes mediates interbacterial antagonism. *Cell Host & Microbe, 16*(2), 227-236. doi:10.1016/j.chom.2014.07.007

Sader, H. S., Fey, P. D., Limaye, A. P., Madinger, N., Pankey, G., Rahal, J., . . . Jones, R. N. (2010). Evaluation of vancomycin and daptomycin potency trends (MIC creep) against methicillin-resistant *Staphylococcus aureus* isolates collected in nine U.S. medical centers from 2002 to 2006. *Antimicrobial Agents and Chemotherapy, 54*(3), 1383. doi:10.1128/AAC.01503-09

Sauert, M., Temmel, H., & Moll, I. (2015). Heterogeneity of the translational machinery: Variations on a common theme. *Biochimie, 114*, 39-47. doi:10.1016/j.biochi.2014.12.011

Schneewind, O., & Missiakas, D. (2014). Genetic manipulation of *Staphylococcus aureus*. *Current Protocols in Microbiology, 32*(1), 9C.3.1-9C.3.19. doi:10.1002/9780471729259.mc09c03s32

Silhavy, T. J., Kahne, D., & Walker, S. (2010). The bacterial cell envelope. *Cold Spring Harbor Perspectives in Biology, 2*(5), a000414. doi:10.1101/cshperspect.a000414

Sjödin, A., Svensson, K., Ohrman, C., Ahlinder, J., Lindgren, P., Duodu, S., . . . Forsman, M. (2012). Genome characterisation of the genus *Francisella* reveals insight into similar evolutionary paths in pathogens of mammals and fish. *BMC Genomics, 13*(1), 268. doi:10.1186/1471-2164-13-268

Sjöstedt, A. (2007). Tularemia: History, epidemiology, pathogen physiology, and clinical manifestations. *Annals of the New York Academy of Sciences, 1105*(1), 1-29. doi:10.1196/annals.1409.009

Su, J., Yang, J., Zhao, D., Kawula, T. H., Banas, J. A., & Zhang, J. R. (2007). Genome-wide identification of Francisella tularensis virulence determinants. *Infection and immunity*, *75*(6), 3089–3101. https://doi.org/10.1128/IAI.01865-06

Takada, H., Morita, M., Shiwa, Y., Sugimoto, R., Suzuki, S., Kawamura, F., & Yoshikawa, H. (2014). Cell motility and biofilm formation in *Bacillus subtilis* are affected by the ribosomal proteins, S11 and S21. *Bioscience, Biotechnology, and Biochemistry*, *78*(5), 898-907. doi:10.1080/09168451.2014.915729

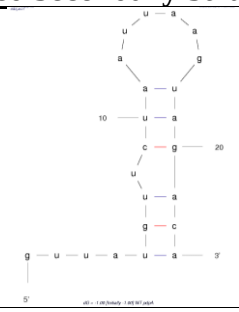
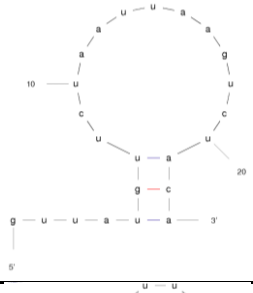
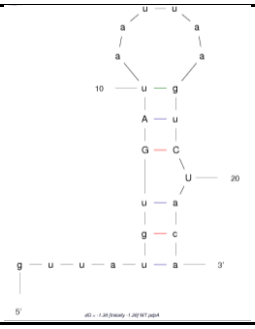
Van Duin, J., & Wijnands, R. (1981). The function of ribosomal protein S21 in protein synthesis. *European Journal of Biochemistry, 118*(3), 615. doi:10.1111/j.1432-1033.1981.tb05563.x

Williams, D. H., & Bardsley, B. (1999). The Vancomycin Group of Antibiotics and the Fight against Resistant Bacteria. *Angewandte Chemie (International ed. in English)*, *38*(9), 1172–1193. https://doi.org/10.1002/(SICI)1521-3773(19990503)38:9<1172::AID-ANIE1172>3.0.CO;2-C

Xue, S., & Barna, M. (2012). Specialized ribosomes: A new frontier in gene regulation and organismal biology. *Nature Reviews. Molecular Cell Biology, 13*(6), 355. doi:10.1038/nrm3359

Appendix 1: Supplemental Tables and Figures

Supplemental Table 1. Proposed mutations to 5´ UTR of *pdpA* and effects on predicted mRNA secondary structure.

Mutations	Sequence*	Predicted Secondary Structure**
None (wild-type)	gttatgttctaattaagtagaca	
A19C G20T	gttatgttctaattaagtCTaca	
A19C G20T T8G C9A	gttatgtGAtaattaagtCTaca	

* Capitalized letters indicate nucleotide changes compared to wild-type.

**Secondary structures predicted using mfold software from the University of Albany.

A)

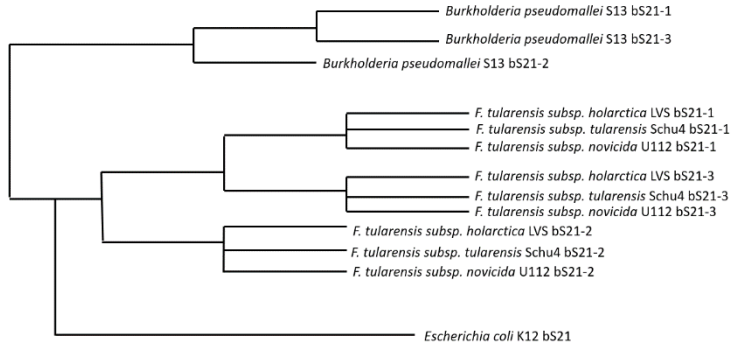
```
bS21-1    MLSIRVDEHKPFDISLRNFKRACEKAGIKQELRDRQHYVKPTEKRKIAKRQAVKRARISQRRAFI-
bS21-2    MPSVRIKEREPFDVALRRFKRSCEKAGIVSELRRREYFEKPTWARKRKKTAAVKRAHKSNIIVKR-
bS21-3    MPRIIVDPKKPFDISLRNFKRACEKAGIKQELRDRQHYVKPTQKRKIAKKAAISKAKKEARRSYSY
```

Identical amino acids
Similar properties

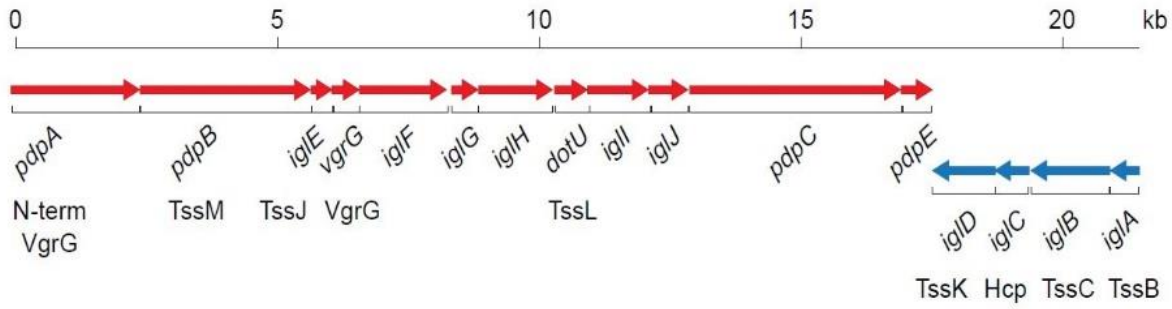
B)

	bS21-1	bS21-2	bS21-3
bS21-1	100.00	52.31	72.31
bS21-2	52.31	100.00	46.15
bS21-3	72.31	46.15	100.00

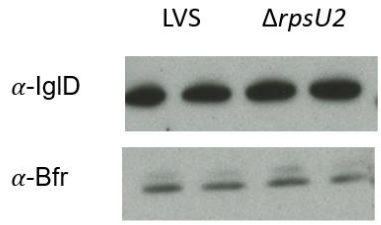
Supplemental Figure 1. Alignment and percent identity matrix of bS21 paralogs in *F. tularensis*. a) Amino acid alignment from Clustal Omega multiple sequence alignment tool. Amino acids highlighted in blue are identical amongst all three paralogs, amino acids highlighted in gray have strong similarity amongst all three homologs. b) Matrix showing percent identify at the amino acid level of bS21 paralogs from Clustal Omega Multiple sequence alignment tool.



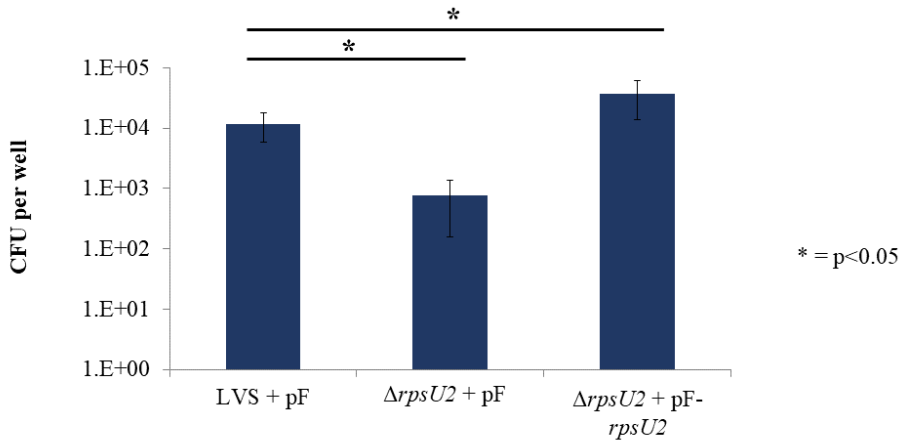
Supplemental Figure 2. Reduced phylogenetic tree comparing amino acid sequences of bS21 in bacterial species. All *F. tularensis* species cluster together, suggesting these are the results of gene duplication events.



Supplemental Figure 3. Diagram of the *Francisella* Pathogenicity Island (FPI) which encodes the T6SS. Gene names and protein products homologous to conserved T6SS in other bacteria are indicated.



Supplemental Figure 4. Bfr protein abundance is unaffected by loss of *rpsU2*. Immunoblots of whole cell lysates from wild-type *F. tularensis* and Δ*rpsU2* in biological duplicate, with specified antibodies. IglD serves as a loading control.



Supplemental Figure 5. Loss of *rpsU2* leads to an intramacrophage growth defect in *F. tularensis*. Results of an intramacrophage growth assay of indicated bacterial cells in J774A.1 macrophage cells. Bacterial growth after 24 hours is shown here. pF is the empty vector.

Appendix 2: Detailed Methods

1. Bacterial strains and growth conditions

Unless otherwise noted, we will grow our bacterial strains as indicated here. We will grow *Francisella tularensis subsp. holarctica* Live Vaccine Strain (LVS) in supplemented Mueller-Hinton broth (0.025% iron pyrophosphate, 0.1% glucose, and 2% Isovitalex), shaking aerobically or on cystine heart agar plates with 1% hemoglobin at 37˚C. We will grow *Staphylococcus aureus* HG003 in tryptic soy broth (TSB) shaking aerobically or on tryptic soy agar (TSA) plates at 37˚C.

2. β-galactosidase assays

As described in Dove & Hochschild (2004), we will grow *F. tularensis* containing reporter constructs to mid-log phase. We will chill the cells on ice to stop growth and will read optical densities at A_{600}. We will add cells to the assay buffer, containing 0.06M Na_2HPO_4, 0.04M NaH_2PO_4, 0.01M KCl, 0.001M $MgSO_4$, and 0.05M β-mercaptoethanol. We will add SDS to a final concentration of 0.003% and chloroform to a final concentration of 6% to permeabilize the cells. We will vortex the cells, acclimate to a 28˚C water bath, then add ONPG to a final concentration of 0.67 mg/mL. ONPG is a substrate for the β-galactosidase enzyme and one of the products of this enzymatic reaction, o-nitrophenol, produces a yellow color. After significant yellow color is produced, we will stop the reaction using 1M Na_2CO_3 and record the reaction time. We will then analyze each sample via spectrophotometer at A_{420} and A_{550}. We will calculate Miller units using the following equation: Miller Units = $1000 \times [(OD_{420} - 1.75 \times OD_{550})/(t \times v \times OD_{600})]$, where t=time of reaction in minutes and v=volume of culture originally used.

3. Ribosome purification

We will purify 70S ribosomes based on a protocol adapted from Moazed & Noller (1989). We will grow *F. tularensis* to mid-log in supplemented Mueller-Hinton broth, shaking aerobically

at 37˚C. We will pellet the cells at 4˚C, then wash twice with lysis buffer, consisting of 10 mM HEPES KOH pH 7.6, 10 mM $MgCl_2$, and 50 mM NH_4Cl ($H^{10}M^{10}A^{50}$), and resuspend in the same buffer with 20 U DNase I. Next, we will lyse the cells by passing through a French Press three times at approximately 800 psi. We will spin the lysate in an ultracentrifuge for 15 minutes (4˚C, 80,000 x g) to remove cell debris. We will layer the supernatant on top of a sucrose cushion, comprised of 10 mM HEPES KOH pH 7.6, 10 mM $MgCl_2$, 500 mM NH_4Cl, with 20% sucrose. We will then spin this again in the ultracentrifuge for 4 hours (4˚C, 146,000 x g) to separate ribosomes from other cell materials. We will wash the pellet twice and resuspend in the $H^{10}M^{10}A^{50}$ lysis buffer. We will then layer this suspension onto another sucrose cushion of 10 mM HEPES KOH pH 7.6, 10 mM $MgCl_2$, 50 mM NH_4Cl, with 40% sucrose and spin for 14 hours (4˚C, 146,000 x g) to further purify the ribosomes. Finally, we will resuspend the purified 70S ribosomes in $H^{10}M^{10}A^{50}$ and store aliquots at -80˚C.

4. Deletion of *rpsU* in *S. aureus*

The following protocol is based on the published protocol of Kato & Sugai (2011). First, we will construct a plasmid with approximately 600-base pairs upstream of the *rpsU* gene fused to 600-base pairs downstream of the *rpsU* gene. We will generate the fragments containing DNA specifying appropriate enzyme digestion sites using PCR, digest with SalI and BamHI (NEB), and ligate into a similarly digested pKFT backbone. The backbone of this vector contains, in addition to a multiple cloning site, *ampR*, *tetL*, a constitutive origin for plasmid replication in *E. coli*, and a temperature-sensitive origin for plasmid replication in *S. aureus*. These markers will be crucial for selecting for colonies that contain the plasmid integration.

We will then transform the ligations into *E. coli* XL-1 blue cells via heat shock at 42˚C to propagate the plasmid. After 1 hour of recovery in Luria broth (LB), we will plate the *E. coli* cells

on LB agar with 100 µg/mL of ampicillin to select for cells with the plasmid incorporated, and grow at 37˚C overnight (Agilent manual). We will then purify the plasmids using a miniprep kit from QIAgen, store at -20˚C, and confirm the sequence at the Rhode Island Genomics and Sequencing Center. Once the vector has been confirmed, we must propagate it in *S. aureus* RN4220, a strain that will add modifications to the plasmid to protect it from nucleases in our target strain, *S. aureus* HG003. We will transform the plasmid into *S. aureus* RN4220 and grow at 30˚C on trypticase soy agar (TSA) plates with 3 µg/mL of tetracycline for selection. We will then inoculate colonies in 3 mL TSB (trypticase soy broth) with tetracycline and grow overnight at 30˚C, shaking. We will isolate the plasmid again using the QIAgen miniprep kit. We will electroporate it into *S. aureus* HG003 using the following settings in a 1 mm cuvette: 2.5 kV, 25 µF, and 100 Ω (Schneewind & Missiakis, 2014). We will incubate the cells at 42˚C on TSA plates with tetracycline, as this temperature is non-permissive for the temperature-sensitive origin. We will grow these cells on plates with tetracycline in order to select for cells expressing the tetracycline-resistance gene, which will only be expressed in cells in which the plasmid has been incorporated into the genomic DNA. We will conduct colony PCR to confirm that the plasmid integrated into the correct location, using primers located outside of the *rpsU* gene. We will grow primary integrants shaking overnight at 25˚C in TSB (no antibiotics), then sub-culture into fresh TSB and grow up at 25˚C again. This will facilitate crossing-out of the residual plasmid from the *S. aureus* genome. We will plate cells both on TSA plates and TSA plates with tetracycline. We will screen tetracycline-sensitive colonies using PCR to confirm the deletion of *rpsU* and removal of the plasmid. We will then amplify genomic DNA of the strain for sequencing at the Rhode Island Genomics and Sequencing Center for further confirmation and store in TSB with 15% glycerol at -80˚C.

5. Population analysis

The population analysis protocol is adapted from Hanaki & Hiramatsu (2001). We will first grow our wild-type and *ΔrpsU* strains in TSB at 37˚C, shaking overnight. We will normalize the optical density of cell cultures to A_{578}=0.3 to assure all plates start with the same number of colony-forming units (CFUs, approximately 10^8). We will then ten-fold serially-dilute the cells six times in TSB, such that the lowest concentration will have approximately 10^2 CFUs. We will prepare brain heart infusion (BHI) plates with 1:2 serial dilutions of each antibiotic. For vancomycin, the published minimum inhibitory concentration (MIC) of clinical isolates of MRSA was approximately 0.625 μg/mL, while the daptomycin MIC ranged from 0.156 to 0.219 μg/mL (Sader et al., 2010). Therefore, we will prepare 8 serial dilutions starting at 10 μg/mL, down to 0.078 μg/mL for vancomycin, and starting at 5 μg/mL down to 0.039 μg/mL for daptomycin. Because antibiotics will be incorporated into agar plates, we can easily adjust concentrations based on the results of the first assay, without the need for step-wise serial dilutions. For instance, we may find it is more informative to look at concentrations shifting by only 0.1 μg/mL (i.e. 1 μg/mL, 0.9 μg/mL, 0.8 μg/mL, and so on). We will sterilely spread each dilution of cells onto plates of each antibiotic concentration in triplicate. We will leave plates with bacteria on the benchtop for 15 minutes to dry, thereby preventing patchy and unreliable growth. We will incubate the plates at 37˚C for 48 hours before counting colonies. We will then determine the MIC as the lowest concentration of antibiotic that resulted in no colonies. We expect, based on previous studies, that Δ*rpsU* cells will have a higher MIC for both daptomycin and vancomycin when compared to wild-type *S. aureus.*

6. TEM preparation

We will prepare TEM samples based on descriptions in Hanaki et al. (1998). First, we will grow wild-type and Δ*rpsU* cells in BHI to logarithmic phase at 37˚C, shaking aerobically. We will

then fix the cells by incubating for two hours in 2% glutaraldehyde in 0.1 M sodium phosphate buffer, pH 7.4. We will stain the cells by incubating for 2 hours at 4˚C with 1% osmium tetroxide, then dehydrate with ethanol and embed in resin, preparing thin sections. We will next stain the sections with uranyl acetate and lead citrate, before analyzing under a transmission electron microscope (Hanaki et al., 1998). We will set the magnification to x30,000 in order to measure cell wall thickness (Cui et al., 2006).