A ribosomal protein homolog regulates gene expression and virulence in a bacterial pathogen

Hannah S. Trautmann[1] and Kathryn M. Ramsey[1,2,*]

[1]Department of Cell and Molecular Biology, University of Rhode Island, Kingston, RI 02881, USA

[2]Department of Biomedical and Pharmaceutical Sciences, University of Rhode Island, Kingston, RI 02881, USA

* To whom correspondence should be addressed: Kathryn M. Ramsey kramsey@uri.edu

Abstract

There are numerous mechanisms by which bacteria can control gene regulation post-transcriptionally. The molecular machine necessary for protein synthesis, the ribosome, is generally considered constitutively functioning and lacking any inherent regulatory capacity. Yet ribosomes are commonly heterogenous in composition and the impact of ribosome heterogeneity on translation and the regulation of translation is not well understood. Here we determine that changes in ribosome protein composition regulate gene expression in the intracellular bacterial pathogen *Francisella tularensis*. Because *F. tularensis* encodes three distinct homologs for bS21, a ribosomal protein involved in translation initiation, we reasoned that cells might contain ribosomes that differ with respect to which bS21 homolog is incorporated. Our analysis of purified ribosomes reveals that *F. tularensis* ribosomes are in fact heterogenous with respect to bS21. When *F. tularensis* cells lack one of these bS21 homologs, bS21-2, there are significant changes to the cellular proteome that cannot be explained by changes in mRNA abundance. These findings are consistent with bS21-2 functioning either directly or indirectly as a post-transcriptional regulator of gene expression. Among the differentially abundant proteins are the type VI secretion system (T6SS) proteins encoded by the Francisella Pathogenicity Island. This reveals that bS21-2 is a novel positive post-transcriptional regulator of *F. tularensis* genes that are absolutely essential for intramacrophage replication. Consistent with bS21-2 functioning as a positive regulator of virulence genes, loss of bS21-2 leads to an intramacrophage growth defect. Although bS21-2 and another homolog, bS21-3, can complement the loss of bS21-2 with respect to T6SS protein abundance, bS21-2 is uniquely necessary for robust intramacrophage growth, suggesting that bS21-2 regulates additional gene(s) key for virulence, distinct from the T6SS. Together, these results indicate that in *F. tularensis*, ribosome composition modulates

gene expression and virulence. Our findings are consistent with a model in which bS21 homologs function as post-transcriptional regulators of gene expression, allowing preferential translation of specific subsets of mRNAs, likely at the stage of translation initiation. This work also raises the possibility that bS21 in other organisms may function similarly and that ribosome heterogeneity may be a mechanism by which many bacteria post-transcriptionally regulate gene expression.

Importance

There are many and diverse ways in which bacteria post-transcriptionally regulate gene expression to quickly modify the proteome. However, while bacterial ribosomes are commonly heterogenous in composition (e.g., incorporating different homologs for a ribosomal protein), how heterogeneity impacts translation and gene regulation is unclear. Here we determine that the intracellular human pathogen *Francisella tularensis* has heterogenous ribosomes, incorporating one of three homologs for the ribosomal protein bS21. Furthermore, one bS21 homolog functions, either directly or indirectly, as a post-transcriptional regulator of gene expression and regulator of the *F. tularensis* type VI secretion system, an essential virulence factor. This bS21 homolog is additionally uniquely important for robust intracellular growth. Our data support a model in which bS21 heterogeneity leads to modulation of translation and post-transcriptional gene regulation. Regulation of translation by bS21, or another source of ribosomal heterogeneity, may be a conserved mechanism to control gene expression across the bacterial phylogeny.

Introduction

Regulation of translation provides bacteria with a rapid way to modify gene expression. While many distinct mechanisms permit this fine-tuning (1, 2) the impact of ribosome composition on gene expression remains poorly-understood. In bacteria, ribosomes are diverse and commonly heterogenous with respect to ribosomal protein (r-protein) content, post-translational modifications, rRNA content, or post-transcriptional modifications (reviewed in 3). The functional consequences of ribosome heterogeneity are unclear but may include the formation of "specialized ribosomes," or ribosomes with altered activity due to their distinct composition (4). Although specialized ribosomes are not well described in bacteria, exciting recent studies have connected altered rRNA content of ribosomes and gene regulation (5, 6) and, in *Mycobacterium smegmatis*, ribosomes containing alternate r-protein homologs translate some genes with differential efficiency (7).

Francisella tularensis is a Gram-negative, facultative intracellular bacterium that causes the potentially fatal human disease tularemia (8). After internalization into host cells, *F. tularensis* must escape from the Francisella-containing phagosome to replicate inside the cytosol. This escape process requires a type VI secretion system (T6SS), which modifies the host cell by delivery of effector proteins (9–12). Production of this T6SS is coordinately regulated by the transcription factors MglA, SspA, and PigR, as well as the signaling molecule ppGpp (13–20). Regulation of the T6SS is arguably the most well-understood virulence regulatory network in *F. tularensis*. However, much remains to be learned about the regulation of other virulence factors.

Despite its relatively small genome (< 2 Mbp), *F. tularensis* encodes three distinct *rpsU* genes (*rpsU1*, *rpsU2*, and *rpsU3*), which encode homologs of the small ribosomal subunit protein bS21 (bS21-1, bS21-2, and bS21-3, respectively). This is the only apparent source of ribosome heterogeneity in *F.*

tularensis, as the three rRNA operon sequences are identical and no other r-proteins are encoded by multiple homologs. In *Escherichia coli*, bS21 is involved in translation initiation (21, 22) and, consistent with this activity, is found on the ribosome close to the anti-Shine-Dalgarno sequence near the mRNA exit channel (23, 24). Furthermore, bS21 is one of the last r-proteins to assemble into the ribosome, is considered "loosely associated," and is easily exchanged among assembled ribosomes (25, 26).

Using mass spectrometry and immunoblot analyses, we show that ribosomes in *F. tularensis* are heterogenous with respect to bS21 content and can incorporate any of the three bS21 homologs into actively-translating ribosomes. Using quantitative whole-cell proteomics, quantitative immunoblots, and transcriptomic analyses, we demonstrate that loss of a particular bS21 homolog, bS21-2, leads to changes in abundance for a subset of proteins that cannot be explained by changes in transcript abundance. Among the regulated proteins are multiple virulence factors, including those that comprise the T6SS. Finally, using intramacrophage growth assays, we provide evidence that bS21-2, and not the other bS21 homologs, promotes intramacrophage growth. Our findings reveal that a specific r-protein homolog in *F. tularensis*, bS21-2, regulates gene expression at the level of protein abundance and is a positive regulator of virulence.

Results

Francisella species encode three bS21 homologs

The genomes of multiple Francisella species contain three distinct genes encoding bS21 (*rpsU1*, *rpsU2,* and *rpsU3*), raising the possibility that cells contain ribosomes that are heterogenous with respect to bS21 content. The gene encoding one homolog in *F. tularensis*, *rpsU2* (encoding bS21-2), is

84 syntenic with the single bS21-encoding gene in *Escherichia coli* (**Figure S1**). In *E. coli*, *rpsU* is the first in
85 an operon referred to as the macromolecular synthesis operon, encoding key proteins for initiation of
86 translation (bS21), DNA replication (DNA primase), and transcription (RNA polymerase σ^{70}) (27). The
87 corresponding operon in Francisella species including *F. tularensis* also contains *yqeY*, which may
88 encode a protein necessary for correct tRNA aminoacylation (28). Another bS21 homolog, bS21-1, is
89 encoded by *rpsU1* in an apparent operon downstream of the gene for cold shock protein CspC. There
90 are no annotated genes in the same transcriptional context as *rpsU3*, the gene encoding the third
91 homolog, bS21-3. The bS21 homologs in *F. tularensis* are distinct but similar, with amino acid identities
92 ranging from 48 – 72%, and are similar to *E. coli* bS21 (51 – 60% identical, with bS21-2 having the
93 highest identity; **Figure S2**).

94 **F. tularensis *ribosomes are heterogenous***

95 The presence of three distinct genes encoding bS21 raises the potential for *F. tularensis*
96 ribosomes to be heterogenous with respect to bS21. To investigate this possibility, we used sucrose
97 cushion centrifugation to isolate ribosomes from *F. tularensis* LVS grown *in vitro* in quadruplicate and
98 analyzed their protein composition using liquid chromatography tandem mass spectrometry (LC-
99 MS/MS). Approximately 80% of the spectral counts corresponded to ribosomal proteins or proteins
100 associated with transcription and translation complexes (e.g., RNA polymerase, translation release
101 factors, SRP), indicating *F. tularensis* ribosomes purified in this manner are highly pure (**Figure 1A**,
102 **Table S1**). Despite the small size of bS21 (approx. 8 kDa), we identified multiple peptides corresponding
103 to bS21-2 in all samples. In one sample, peptides shared between bS21-1 and bS21-3 were detected
104 (**Figure 1B**). This suggests that bS21-2 is the most abundant homolog in wild-type cells, consistent with

its production from an operon encoding proteins essential for transcription and DNA replication. It also suggests that either bS21-1, bS21-3, or both, are incorporated into ribosomes in LVS. However, it does not allow us to determine the next-most abundant homolog (bS21-1 or bS21-3) or confirm incorporation of both of these other homologs. Regardless, these results demonstrate that multiple bS21 homologs are incorporated into wild-type *F. tularensis* ribosomes and that ribosomes in *F. tularensis* are heterogenous, containing different bS21 homologs.

We next wanted to determine if each bS21 homolog can be found in actively-translating ribosomes. To track each bS21, we modified each homolog to encode a C-terminal vesicular stomatitis virus glycoprotein (VSV-G) tag and ectopically expressed them individually from a plasmid , using the same promoter, in wild-type cells. Lysate fractions of these cells were analyzed by immunoblotting after sucrose gradient sedimentation (**Figure 1C**; **Figure S3**). When ectopically expressed (rather than produced from its native locus), bS21-1 was the least abundant homolog while bS21-3 was produced at the highest level. Each homolog was found in fractions corresponding to the 30S, 70S, and polysomes. Although bS21 is thought to function primarily in translation initiation, our findings indicate that each bS21 homolog associates with the ribosome throughout the translation cycle.

Loss of bS21-2 leads to changes in protein, not transcript, abundance

Because the ribosomal protein bS21 is involved in translation initiation, we hypothesized that loss of a bS21 homolog may impact translation and result in changes in abundance in a subset of proteins. To test this hypothesis, we individually deleted each of the three genes encoding bS21 homologs. This led us to determine that no single bS21 homolog is essential for cell growth. We subsequently grew wild-type cells and cells lacking single bS21 homologs to mid-log *in vitro* and used

data-independent acquisition (DIA) mass spectrometry analysis (29) to compare relative protein abundance in cell lysates. Using this method, 68% of the total proteins predicted to be encoded by *F. tularensis* LVS were identified and analyzed (1194 of 1754). When compared to wild-type, we did not detect any significant changes in protein abundance in cells lacking either of the two lower-abundance bS21 homologs, bS21-1 and bS21-3 (>1.5-fold altered with an adjusted p-value <0.05, excluding bS21). In contrast, cells lacking the most abundant homolog, bS21-2 (Δ*rpsU2*), had significant proteomic differences compared to wild-type cells. Specifically, we found 185 unique proteins (~16% of detected proteins) have altered abundance in cells without bS21-2 compared to wild-type cells (**Figure 2**, data on y-axis, **Table S2**).

To determine if these changes in protein abundance can be explained by corresponding changes in transcription, we performed transcriptomic analyses on wild-type cells, cells lacking bS21-2 (Δ*rpsU2*), and cells lacking the native bS21-2 but ectopically expressing bS21-2-V from a plasmid. Comparing cells with and without native bS21-2, we identified 105 differentially expressed genes (>2-fold altered with an adjusted p-value <0.05, excluding *rpsU*; **Figure 2**, data on x-axis, **Table S3**). All of these changes were complemented by ectopic expression of bS21-2-V on a plasmid.

Our analysis revealed that in cells lacking bS21-2, the largest change in transcript abundance is a six-fold increase in *yqeY*, the gene directly downstream from *rpsU2* (which encodes bS21-2). This increase in transcript abundance is complemented by ectopic expression of bS21-2-V, suggesting that bS21-2 functions as a negative regulator of its own operon. Translational feedback regulation is well-established for multiple ribosomal proteins but, to the best of our knowledge, this is the first report of

translational regulation of ribosomal proteins in *F. tularensis* and the first report that bS21 regulates its own production (30, 31).

Comparison of our proteomic and transcriptomic analyses reveals that the changes in protein abundance are not generally due to changes in transcript abundance. Of the 185 differentially abundant proteins in cells lacking bS21-2, only ~12% (23) can be explained by altered transcription (**Figure 2**, yellow dots), while about 88% (162; **Figure 2**, blue dots and orange dot) have changes in protein abundance without a corresponding change in transcript abundance. These discrepancies between transcript abundance and protein abundance support a model in which bS21-2 controls expression, either directly or indirectly, of some genes at the level of translation.

bS21-2 controls the abundance of type VI secretion system proteins, which are essential for virulence

Among the proteins with altered abundance in cells lacking bS21-2, we identified twelve out of sixteen proteins encoded on the Francisella pathogenicity island (FPI). The FPI encodes a unique type VI secretion system (T6SS) that is absolutely essential for intramacrophage growth and virulence of *F. tularensis* (32–34). Using quantitative immunoblotting and antibodies specific to a subset of *F. tularensis* T6SS proteins, we validated that cells lacking bS21-2 have differences in those T6SS proteins (**Figure 3**). Consistent with the mass spectrometry results, we found reductions in virtually all probed T6SS proteins, including an ~4-fold reduction in PdpB, the TssM/IcmF homolog. Using this approach, we also found an ~2.4-fold reduction in IglA and ~1.7-fold reduction in IglB, T6SS proteins that are just below the cutoff for statistical significance in our mass spectrometry analysis. Since we identified this differential abundance using a more sensitive method of comparison, it raises the possibility that all FPI-encoded proteins may be differentially abundant in cells lacking bS21-2

compared to wild-type cells, but we do not have antibodies specific to the remaining proteins (i.e., PdpE and VgrG) to test this hypothesis. Also consistent with our mass spectrometry findings, IglD (the homolog of TssK) is the only T6SS protein with increased, rather than decreased, protein abundance (**Figure 3**). Each of these changes in protein abundance can be complemented by ectopic expression of bS21-2-V, driven by the *groES* promoter on a plasmid (**Figure 3**).

These changes in protein abundance likely reflect positive regulation of most, but not all, T6SS proteins by bS21-2 at the level of translation, either directly or indirectly. Our findings are inconsistent with bS21 positively regulating transcription; it is well-established that transcription of FPI operons are coordinately controlled and our RNA-Seq analysis reveals that cells lacking bS21-2 do not have FPI-wide transcript reductions (**Table S4**) (13–16, 20, 35). In a complementary approach, we compared the transcript abundance for specific FPI genes using quantitative RT-PCR and included cells lacking PigR, a transcription factor critical for positive transcriptional regulation of FPI genes (14–16, 20, 35)(**Figure S4**). We confirmed that cells lacking PigR have major decreases in FPI transcript abundance but cells lacking bS21-2 do not have compelling (2-fold or greater) changes in FPI transcript abundance or in transcript abundance of the positive regulator PigR, consistent with the RNA-Seq results. We considered the possibility that loss of bS21-2 could indirectly impact T6SS protein abundance by altering protein stability, but the half-life of one of the most differentially regulated proteins, PdpB, was unchanged in cells with and without bS21-2 (longer than 120 minutes, **Figure S5**). Our results are consistent with bS21-2 controlling expression of T6SS proteins at the level of translation.

Other bS21 homologs impact the abundance of type VI secretion system proteins

Our findings indicate that bS21-2 is the most abundant bS21 homolog in wild-type cells of LVS. However, it is not clear if the majority of ribosomes in cells lacking bS21-2 incorporate another bS21 homolog or no bS21 at all. This leads to the question: do all bS21 homologs regulate T6SS protein translation or does bS21-2 specifically control translation of T6SS proteins? To answer this question, we ectopically expressed either bS21-1-V or bS21-3-V in cells lacking bS21-2, similarly to the ectopic expression of bS21-2-V. We subsequently used quantitative immunoblot analyses to assess the abundance of each ectopically expressed bS21 homolog and a subset of T6SS proteins (**Figure 3**). While this strategy resulted in comparable amounts of bS21-2 and bS21-3, ectopic expression results in approximately 2-fold less bS21-1 than the other homologs, consistent with its lower expression in wild-type cells (**Figure 3, Figure 1**). With respect to T6SS protein abundance, ectopic expression of bS21-3 restores all probed proteins to wild-type levels, complementing the loss of bS21-2 (**Figure 3**). However, bS21-1 does not appear to complement T6SS protein production completely (**Figure 3**). This may be due to reduced levels of bS21-1, lack of specific ability to regulate of T6SS proteins, or a combination of the two factors. Notably, loss of bS21-2 results in a growth defect (**Table S5**) that can be complemented by ectopic expression of bS21-2 or bS21-1, but not bS21-3. That cells lacking bS21-2 with ectopic expression of bS21-3 have wild-type levels of T6SS proteins and yet still have a growth defect reveals that changes in T6SS proteins are not due simply to changes in growth rate. Our findings allow us to conclude that incorporation of either bS21-2 or bS21-3 – and to a lesser extent, bS21-1 – into ribosomes regulates production of T6SS proteins.

bS21-2 is important for intramacrophage growth

A functional T6SS is essential for *F. tularensis* intramacrophage replication and is a strict requirement for virulence (32–34). The observed differences in FPI protein abundance led us to hypothesize that T6SS function may be compromised in cells lacking bS21-2 and these cells may be attenuated for intramacrophage growth. We tested the ability of cells lacking bS21-2 (Δ*rpsU2*) to survive in murine macrophage-like J774A.1 cells. This revealed a significant defect in the ability of bS21-2 mutant cells to replicate in macrophage; we recovered ten-fold fewer bS21-2 mutant bacteria after 24 hours compared to wild-type (**Figure 4**). The intramacrophage growth defect of cells lacking bS21-2 can be restored by ectopic expression of bS21-2 from a plasmid (**Figure 4**). This is in contrast to ectopic expression of bS21-1 and bS21-3, neither of which restores the intramacrophage growth of cells lacking bS21-2 (**Figure 4**). These results indicate that bS21-2 is specifically required for intramacrophage survival, despite the fact that ectopic expression of bS21-1 restores *in vitro* growth rates and ectopic expression of bS21-3 restores T6SS protein production *in vitro* (**Figure 3, Table S5**).

In summary, only the presence of bS21-2, not bS21-1 or bS21-3, can restore the intramacrophage growth defect of cells without bS21-2. This reveals that bS21-2 is critical for *F. tularensis* virulence and fits a model in which bS21-2 specifically regulates one or more genes necessary for intramacrophage growth in addition to T6SS genes, a topic still under investigation.

Discussion

The findings described here reveal that ribosome composition in *F. tularensis* is heterogenous with respect to the small ribosomal protein bS21 and that this heterogeneity impacts regulation of gene expression at the level of translation. In particular, by studying cells that contain ribosomes either with or without one of the three bS21 homologs, we have identified bS21-2 as a positive regulator of

most of the T6SS proteins. Additionally, cells lacking bS21-2 are defective for intramacrophage growth; since this defect can only be complemented by bS21-2, even though bS21-3 and (to a lesser extent) bS21-1 can restore T6SS protein abundance, this intramacrophage growth defect may be independent of the impact bS21-2 has on the T6SS. This allows us to conclude that bS21-2 is important for the intramacrophage growth of *F. tularensis*, potentially by regulating the translation of one or more proteins (in addition to the T6SS), necessary for virulence.

Our approach in studying bS21 homologs in *F. tularensis* has thus far focused on the homolog bS21-2, whose loss led to phenotypic change. Our data suggest that bS21-2 is the most abundant homolog in the conditions studied. We hypothesize that cells without bS21-1 and bS21-3 did not exhibit distinct phenotypes under the conditions of our experiments due to their relatively low abundance. Both of these homologs may also regulate gene expression under conditions when they are more abundant, but these conditions are not yet identified. Additionally, in our study of cells without bS21-2, it is not clear if the majority of ribosomes lack bS21 entirely or instead incorporate bS21-1 or bS21-3; our findings only extend to heterogeneity with respect to the presence or absence of bS21-2.

Comparison of *rpsU* genes across the bacterial phylogeny reveals that many clades and species do not encode bS21, suggesting that it is not essential for translation (36, 37). However, targeted deletion of the single *rpsU* gene in *E. coli* has not been successful, suggesting bS21 is essential in *E. coli* (38–40). We reported in previous work that the *F. tularensis* homolog syntenic with *E. coli rpsU*, *rpsU2*, is essential *in vitro* using transposon-insertion sequencing (Tn-Seq) (41). Yet using a targeted allelic exchange approach, we have been able to successfully delete each *rpsU* homolog individually,

indicating that none of the bS21 homologs is individually essential. Our identification of *rpsU2* as an essential gene was likely due to the polar effects of transposon insertion into the first gene of an operon containing other known essential genes (*dnaG*, encoding primase, and *rpoD*, encoding the σ^{70} subunit of RNA polymerase). It is unclear if *F. tularensis* cells lacking all three *rpsU* genes are viable.

The literature reflects that bS21 may regulate gene expression in other bacteria. A recent study of the *Flavobacterium johnsoniae* ribosome revealed that bS21 plays a role in sequestering the anti-Shine-Dalgarno sequence (42). This occlusion occurs through contacts with the C-terminal region of bS21 that are conserved across Bacteroidetes species and provides a rationale to explain why most Bacteroidetes mRNAs lack Shine-Dalgarno sequences. Notably, the mRNA encoding bS21 in *F. johnsoniae* encodes a perfect Shine-Dalgarno sequence, strongly suggesting that bS21 regulates its own expression through translational autoregulation (42). *F. tularensis*, however, is a member of the Gammaproteobacteria, has bS21 homologs that exhibit significant differences from *F. johnsoniae* at the C-terminal region, and encodes mRNAs that commonly contain sequences similar to the consensus Shine-Dalgarno sequence. This suggests that in *F. tularensis*, bS21 exerts its effects on gene expression in a different manner.

In other bacteria that encode it, loss of bS21 leads to a variety of phenotypic changes. In *B. subtilis*, loss of bS21 results in biofilm and motility defects (43) and in *Listeria monocytogenes*, inactivation of bS21 is linked to stress resistance and altered transcript abundance(44, 45). *Staphylococcus aureus* lacking functional bS21 exhibit increased resistance to the antibiotics daptomycin and vancomycin (46–48). Both *Burkholderia pseudomallei* and *F. tularensis* encode multiple bS21 homologs and in both organisms, virulence screens using transposon mutagenesis have

identified one homolog as important for virulence (49, 50). Together, these findings suggest that bS21 may regulate gene expression in diverse bacterial species.

The idea that bS21 might modulate translation for a subset of mRNAs is further supported by the recent discovery that bS21 is encoded by thousands of sequenced bacteriophage genomes and is one of the most commonly encoded phage ribosomal proteins (51, 52). Transcripts encoding bS21 have been detected in metatranscriptomic samples along with transcripts for late-stage replication proteins (53) and at least one phage-encoded bS21 can be incorporated into *E. coli* ribosomes (51). All of this raises the possibility that incorporation of a viral bS21 into the host ribosome may co-opt the translation machinery in favor of viral proteins and replication.

Our work, together with these earlier findings, strongly suggests that incorporation of bS21 into the ribosome can impact translation of a subset of mRNAs. Considering that bS21 can easily be exchanged among ribosomes, this provides an excellent mechanism to quickly fine-tune the cellular proteome. While the molecular mechanism leading to the modulation of translation has yet to be identified, it is reasonable to speculate that bS21 impacts translation during initiation through specific interactions with the 5' untranslated regions of a specific set of mRNAs. These findings also support the idea that changes in ribosome composition may impact translation and provide another source for bacterial control of gene expression.

Materials and Methods

289 ***Bacterial strains and growth conditions***

290 Unless otherwise noted, bacterial strains were grown as indicated here. *Francisella tularensis* subsp.
291 *Holarctica* Live Vaccine Strain (LVS) cells were grown in Mueller-Hinton broth (BD Difco) supplemented
292 with 0.025% iron pyrophosphate, 0.1% glucose, and 2% Isovitalex (sMHB), shaking aerobically or on
293 cystine heart agar plates with 1% hemoglobin (CHA-H) at 37˚C. *Escherichia coli* XL1-Blue cells were
294 grown in lysogeny broth (LB) shaking aerobically or on LB agar plates at 37˚C. Kanamycin was used at
295 concentrations of 5 μg/mL (*F. tularensis)* or 50 μg/mL (*E. coli)*.

296

297 ***Vector construction***

298 Complementation plasmids for each bS21 homolog were created from a plasmid derived from
299 pFNLTP6 (54), pKL42 (pF-PmrA-V). Specifically, the complementation plasmids produce bS21 homologs
300 with a C-terminal VSV-G epitope under the control of the *F. tularensis groES* promoter. Each *rpsU* gene
301 was amplified using a 5´ primer specifying an EcoRI site and an ideal Shine-Dalgarno sequence (5´-
302 AGGAGG-3´) located six nucleotides upstream from the translation start site. The 3´ primer did not
303 include the native stop codon and included DNA specifying a NotI site. The fragment was cloned into
304 EcoRI/NotI digested pKL42, such that the 3´ end of each *rpsU* is in frame with codons specifying three
305 alanines followed by the VSV-G epitope. The resulting plasmids were pKR6 pF-bS21-1-V, pKR7 pF-bS21-
306 2-V, and pKR8 pF-bS21-3-V. The control plasmid pF is the original pFNLTP6 plasmid (containing the
307 *groES* promoter but not any *rpsU* genes nor the VSV-G epitope).

308

309 The plasmid pEX18kan was modified to generate in-frame deletions of each *rpsU* gene as previously
310 described (14). Flanking regions of ~600 base pairs from both sides of each *rpsU* gene were amplified

by PCR. Primers amplifying the DNA adjacent to each *rpsU* gene included the first three or last three codons of the open reading frame and DNA specifying a NotI site, which also encodes an alanine linker (5´-GCGGCCGCT-3´). The two fragments were cloned into BamHI/KpnI-digested pEX18kan for each *rpsU* gene respectively, yielding pKL122 pEXΔ*rpsU1,* pKR11 pEXΔ*rpsU2,* and pKR12 pEXΔ*rpsU3*; these plasmids were used to construct deletions via allelic exchange as described below.

Strain construction

Deletion strains were constructed by allelic exchange as previously (55). Briefly, competent cells were made by washing *F. tularensis* LVS cells in 10% sucrose and resuspending in an equal volume of 10% sucrose to cells. At least 1 µg of allelic exchange plasmid was electroporated into 50 µL competent cells in 0.2 cm cuvettes with a 2.5 kV pulse. Cells were allowed to recover in 4 – 5 mL sMHB for 4-8 hours at 37°C, shaking. Cells in which a single integration event occurred were selected for on CHA-H plates with kanamycin. These cells were subsequently plated on CHA-H containing 10% sucrose and lacking NaCl, allowing for survival only of cells that had crossed out the non-homologous portion of the vector, including *sacB* and kanamycin resistance gene. Colonies that were sucrose-resistant and kanamycin-sensitive were screened for deletions using PCR. Candidate strains were confirmed by amplification of genomic DNA outside of the flanking regions on each side of the deletion and Sanger sequencing (Rhode Island Genomics and Sequencing Center). Plasmid pKL122 pEXΔ*rpsU1* was used to make LVS Δ*rpsU1,* plasmid pRK11 pEXΔ*rpsU2* was used to make LVS Δ*rpsU2*, and plasmid pKR12 pEXΔ*rpsU3* was used to make LVS Δ*rpsU3*.

Complementation plasmids were electroporated into LVS or LVS Δ*rpsU2* cells as described above and selected for on CHA-H plates with kanamycin.

Immunoblotting

Cells were collected from mid-log cultures (OD_{600} 0.3-0.4) and resuspended in sample loading buffer (SLB: 1X NuPAGE LDS with 50 mM DTT) normalized to OD_{600} and heated at 95˚C for 10 minutes. Cell lysates and fractions were separated by SDS-PAGE on 4-12% Bis-Tris NuPAGE gels in MES or MOPS running buffer (Invitrogen) and transferred to PVDF with the Mini Blot Module transfer system (Invitrogen; 20V for 1 hour on ice) or the Criterion cell for midi gels (BioRad; 60V for 40 minutes on ice) with 1X NuPAGE transfer buffer and 10% methanol. Whole cell lysates were analyzed for total protein with the Invitrogen No-Stain Protein labeling reagent and all membranes were blocked with Odyssey blocking buffer diluted 1:5 in PBS overnight. For each antibody, the linear range of protein detection was determined by plotting sequential dilutions of one lysate from each strain as a standard curve to establish appropriate volume of lysate to load. Membranes were probed with indicated monoclonal antibodies (BEI Resources, diluted 1:1000 in blocking buffer for all antibodies except anti-PdpB, which was diluted 1:250) or the VSV-G epitope (Sigma, diluted 1:2222). Proteins were detected using IRDye 800 CW donkey anti-mouse IgG or donkey anti-rabbit IgG (Li-Cor, diluted 1:10,000). Fluorescence was measured and quantified on the LiCor Odyssey CLx imager and software, and protein abundance was calculated relative to total protein in each lane. Experiments were performed at least twice in biological triplicate and two to three technical replicates.

RNA isolation and qRT-PCR

Cells were collected from mid-log cultures (OD_{600} 0.3-0.4). Nucleic acids were isolated using the Direct-Zol RNA purification kit (Zymo Research) according to the manufacturer's protocol. Purified nucleic acids were treated with RQ1 Dnase (Promega) for 1 hour at 37°C and RNA was purified with the Direct-Zol RNA purification kit. cDNA was synthesized using Superscript III reverse transcriptase (Life Technologies) as previously described (14). qRT-PCR was performed using PowerUp SYBR Green Master Mix (Applied Biosystems) and a Roche LightCycler 480 (University of Rhode Island Genomics and Sequencing Center) essentially as described (14). Transcript abundances of *pdpA*, *pdpB*, *iglA*, and *pigR* were compared to three different control genes (*tul4*, *rpoA1*, and *bfr*) and since all results were similar, relative abundance is reported to *tul4*. Experiments comparing wild-type and *rpsU2* mutant cells were performed three times in biological triplicate; experiment with cells lacking PigR was performed once.

RNA-Seq

Approximately 1.5 µg of RNA isolated as above was sent to the Microbial Genome Sequencing Center (MiGS) for RNA-Seq analysis, in biological triplicate (LVS pF) or duplicate (LVS Δ*rpsU2* pF, LVS Δ*rpsU2* pF-bS21-2-V). After using RiboZero Plus rRNA depletion, libraries were made using Illumina Stranded RNA library preparation and sequenced for a minimum of 12 million paired end reads. Sequencing reads will be available in the National Center for Biotechnology Information Gene Expression Omnibus (GEO). Paired-end sequencing reads were mapped to the *F. tularensis* LVS genome (NCBI RefSeq accession number NC_007880) using bowtie2 version 2.2.4. Reads that mapped to annotated genes were counted using HTSeq version 0.11.2, and analysis of differential gene expression was conducted

using DESeq2 version 1.32.0. Reported genes had a 2-fold-higher or -lower abundance than the wild type, all with an adjusted p-value of 0.05 or lower.

70S ribosome purification

70S ribosomes were isolated using sucrose cushion centrifugation essentially as described (56). Briefly, wild-type *F. tularensis* cells were grown in 500 mL sMHB to mid-log (OD_{600} 0.3-0.4). Cells were chilled on ice for 20 minutes, centrifuged at 11,000 x g for 5 minutes at 4˚C, then washed once with buffer $H^{10}M^{10}A^{1000}$ (10 mM HEPES KOH pH 7.6, 10 mM $MgCl_2$, and 100 mM NH_4Cl) to remove ribonucleases. The pellet was then washed twice with buffer $H^{10}M^{10}A^{50}$ (10 mM HEPES KOH pH 7.6, 10 mM $MgCl_2$, and 50 mM NH_4Cl, with or without 5 mM β-mercaptoethanol [BME]), and resuspended in ~15 mL of $H^{10}M^{10}A^{50}$ with 20 U Dnase I. Cells were lysed by passing through a French press three times at 800 psi and cell debris were removed by centrifugation at 146,000 x g for 15 minutes at 4˚C. Supernatant was incubated with 0.5% Brij58 for 30 minutes and layered on top of $H^{10}M^{10}A^{500}$ + 20% sucrose (10 mM HEPES KOH pH 7.6, 10 mM $MgCl_2$, 500 mM NH_4Cl, 20% sucrose, with or without 5 mM BME). Ribosomes were pelleted by ultracentrifugation in 70 Ti rotor for 4 hours at 146,000 x g at 4˚C. The pellet was washed twice with $H^{10}M^{10}A^{50}$ and gently resuspended in $H^{10}M^{10}A^{50}$. This suspension was then layered onto another sucrose cushion ($H^{10}M^{10}A^{50}$ with 40% sucrose) and centrifuged for 14 hours at 146,000 x g at 4°C to further purify the ribosomes. Purified 70S ribosomes were gently resuspended in ~250 µL of $H^{10}M^{10}A^{50}$ and stored at -80˚C.

LC-MS/MS of purified LVS ribosomes

70S ribosomes from wild-type LVS cells were prepared as described above. Samples were either purified via gel stacking prior to mass spectrometry analysis or maintained in $H^{10}M^{10}A^{50}$ and delivered to the Northwestern Proteomics Core. The proteins were in-gel digested or in-solution digested and liquid chromatography tandem mass spectrometry (LC-MS/MS) analysis was completed based on internal protocols, matching peptides to the *F. tularensis* LVS proteome (NC_007880).

DIA mass spectrometry

Cells were collected from mid-log cultures (OD_{600} 0.3-0.4) and resuspended in Buffer 1 (20 mM KHEPES pH 7.9, 50 mM KCl, 0.5 mM DTT) with protease inhibitor tablets (Complete Mini, EDTA-free, Roche). Cells were lysed by sonication and protein concentration was determined using a BCA protein assay (Pierce). Lysates with concentrations between 620 and 862 µg/mL were used by the University of Arkansas for Medical Sciences (UAMS) Proteomics Core for analysis. Protein extraction and protease digestion was completed according to UAMS internal protocols. Data-independent acquisition (DIA) was completed with the Orbitrap Exploris 480 mass spectrometer.

Polysome purification and sucrose gradient sedimentation

Polysomes were isolated essentially as described (57). *F. tularensis* cells were grown until early log (OD_{600} 0.2-0.25). Liquid cultures were rapidly filtered through 0.2 µm nitrocellulose membranes and transferred to a conical tube filled with liquid nitrogen. Cells were lysed by bead-beating with 650 µL flash frozen lysis buffer (25 mM HEPES pH 7.6, 100 mM NH_4Cl, 10 mM $MgCl_2$, 0.4% Triton X-100, 0.1%

NP-40, 100 U/mL Rnase-free Dnase) using the TissueLyser II (Qiagen) five times (15 Hz, 3 mins). Cell debris was pelleted and the polysome-containing lysates were stored at -80˚C.

Sucrose gradients were prepared using 10 and 55% sucrose solutions in 25 mM HEPES pH 7.6, 100 mM NH_4Cl, 10 mM $MgCl_2$ with the BioComp Instruments 153 Gradient Station (BioComp). Cell lysates were layered onto gradients and centrifuged with the Beckman-Coulter SW40 Ti rotor at 40,000 rpm for 2.5 hr at 4˚C. Gradients were fractionated using the Triax full spectrum flow cell and fractionator (BioComp; 0.2 mm/s, 28 fractions) and A260 was measured every second. Collected fractions were stored at -80˚C. 20 µL of each fraction was combined with 10 µL of sample loading buffer (3X NuPAGE LDS with 50 mM DTT) and immunoblotted as described above.

Intramacrophage replication assays

Intramacrophage growth assays were performed as previously described (55). Briefly, approximately 2.5 x 10^4 cells of murine macrophage-like J774A.1 cells were incubated at 37°C in 5% CO_2 overnight in 96-well plates in DMEM (Invitrogen) supplemented with 10% fetal bovine serum (Gemini Bio-Products; DMEM-F). Macrophage cells were infected with LVS and indicated derivative strains at an MOI of approximately 5 – 10. After two hours, cells were washed twice with PBS and media was replaced with DMEM-F containing 10 µg/mL gentamycin. After 2 or 24 hours of infection, macrophage were lysed for 30 minutes in 1% saponin in PBS and plated for enumeration.

Antibiotic chase experiment

Indicated *F. tularensis* LVS cells were grown to mid-log in liquid culture (OD_{600} 0.3-0.4). Spectinomycin was added to a final concentration of 200 μg/mL. Cells were collected at the indicated time points after antibiotic addition and resuspended in sample loading buffer normalized to OD_{600} at t=0. Immunoblotting was conducted as described above and analysis was conducted using one-phase decay equation on Prism 9 (GraphPad). Data represents two experiments in biological triplicate.

Acknowledgements

For helpful comments on the manuscript, we thank Dr. Simon L. Dove, Dr. Steven T. Gregory, and Dr. Matthew M. Ramsey. For use of shared equipment, we thank Dr. Gregory, Dr. Jodi L. Camberg, and Dr. Niall G. Howlett. We thank the other members of the Ramsey laboratory. We also thank Janet Atoyan and the URI Genomics and Sequencing Center (now Rhode Island INBRE Molecular Informatics Core).

Funding Information

This work was funded by a NIGMS CARTD-COBRE Pilot Project Award (P20GM121344—KMR), a NIGMS/RI-INBRE Early Career Development Award (P20GM103430—KMR), and a Rhode Island Foundation Medical Research Grant (2798_20190602—KMR). This work was supported by the USDA National Institute of Food and Agriculture, Hatch Formula project accession number 1017848.

This material is based upon work conducted at a Rhode Island NSF EPSCoR research facility, the Genomics and Sequencing Center, supported in part by the National Science Foundation EPSCoR Cooperative Agreements 0554548, EPS-1004057, and OIA-1655221. Research was made possible by the use of equipment and services available through the Rhode Island Institutional Development

Award (IDeA) Network of Biomedical Research Excellence from the National Institute of General Medical Sciences of the National Institutes of Health under grant number P20GM103430 through the Centralized Research Core facility and the Molecular Informatics Core (RRID:SCR_017685). LC-MS/MS proteomics were performed by the Northwestern Proteomics Core Facility, supported by NCI CCSG P30 CA060553, instrumentation award (S10OD025194), and the National Resource for Translational and Developmental Proteomics supported by P41 GM108569. DIA proteomics were performed by IDeA National Resource for Quantitative Proteomics, supported by NIGMS R24GM137786.

References

1. Hershey JWB, Sonenberg N, Mathews MB. 2012. Principles of Translational Control: An Overview. Cold Spring Harb Perspect Biol 4:a011528.

2. Duval M, Simonetti A, Caldelari I, Marzi S. 2015. Multiple ways to regulate translation initiation in bacteria: Mechanisms, regulatory circuits, dynamics. Biochimie 114:18–29.

3. Byrgazov K, Vesper O, Moll I. 2013. Ribosome heterogeneity: another level of complexity in bacterial translation regulation. Curr Opin Microbiol 16:133–139.

4. Xue S, Barna M. 2012. Specialized ribosomes: a new frontier in gene regulation and organismal biology. Nat Rev Mol Cell Bio 13:355–69.

5. Kurylo CM, Parks MM, Juette MF, Zinshteyn B, Altman RB, Thibado JK, Vincent CT, Blanchard SC. 2018. Endogenous rRNA Sequence Variation Can Regulate Stress Response Gene Expression and Phenotype. Cell Rep 25:236-248.e6.

6. Song W, Joo M, Yeom J-H, Shin E, Lee M, Choi H-K, Hwang J, Kim Y-I, Seo R, Lee JE, Moore CJ, Kim Y-H, Eyun S-I, Hahn Y, Bae J, Lee K. 2019. Divergent rRNAs as regulators of gene expression at the ribosome level. Nat Microbiol 4:515–526.

7. Chen Y-X, Xu Z, Ge X, Hong J-Y, Sanyal S, Lu ZJ, Javid B. 2020. Selective translation by alternative bacterial ribosomes. Proc Natl Acad Sci U S A 117:19487–19496.

8. Sjöstedt A. 2007. Tularemia: History, Epidemiology, Pathogen Physiology, and Clinical Manifestations. Ann N Y Acad Sci 1105:1–29.

9. Barker JR, Chong A, Wehrly TD, Yu J-J, Rodriguez SA, Liu J, Celli J, Arulanandam BP, Klose KE. 2009. The *Francisella tularensis* pathogenicity island encodes a secretion system that is required for phagosome escape and virulence. Mol Microbiol 74:1459–1470.

10. Bröms JE, Meyer L, Sun K, Lavander M, Sjöstedt A. 2012. Unique substrates secreted by the type VI secretion system of *Francisella tularensis* during intramacrophage infection. PLoS One 7:e50473.

11. Eshraghi A, Kim J, Walls AC, Ledvina HE, Miller CN, Ramsey KM, Whitney JC, Radey MC, Peterson SB, Ruhland BR, Tran BQ, Goo YA, Goodlett DR, Dove SL, Celli J, Veesler D, Mougous JD. 2016. Secreted Effectors Encoded within and outside of the Francisella Pathogenicity Island Promote Intramacrophage Growth. Cell Host Microbe 20:573–583.

12. Ledvina HE, Kelly KA, Eshraghi A, Plemel RL, Peterson SB, Lee B, Steele S, Adler M, Kawula TH, Merz AJ, Skerrett SJ, Celli J, Mougous JD. 2018. A Phosphatidylinositol 3-Kinase Effector Alters Phagosomal Maturation to Promote Intracellular Growth of *Francisella*. Cell Host Microbe 24:285-295.e8.

13. Lauriano CM, Barker JR, Yoon S-S, Nano FE, Arulanandam BP, Hassett DJ, Klose KE. 2004. MglA regulates transcription of virulence factors necessary for *Francisella tularensis* intraamoebae and intramacrophage survival. Proc Natl Acad Sci U S A 101:4246–4249.

14. Charity JC, Costante-Hamm MM, Balon EL, Boyd DH, Rubin EJ, Dove SL. 2007. Twin RNA polymerase-associated proteins control virulence gene expression in *Francisella tularensis*. PLoS Pathog 3:e84.

15. Charity JC, Blalock LT, Costante-Hamm MM, Kasper DL, Dove SL. 2009. Small molecule control of virulence gene expression in *Francisella tularensis*. PLoS Pathog 5:e1000641.

16. Brotcke A, Monack DM. 2008. Identification of *fevR*, a novel regulator of virulence gene expression in *Francisella novicida*. Infect Immun 76:3473–3480.

17. Rohlfing AE, Dove SL. 2014. Coordinate control of virulence gene expression in *Francisella tularensis* involves direct interaction between key regulators. J Bacteriol 196:3516–3526.

18. Ramsey KM, Osborne ML, Vvedenskaya IO, Su C, Nickels BE, Dove SL. 2015. Ubiquitous promoter-localization of essential virulence regulators in *Francisella tularensis*. PLoS Pathog 11:e1004793.

19. Cuthbert BJ, Ross W, Rohlfing AE, Dove SL, Gourse RL, Brennan RG, Schumacher MA. 2017. Dissection of the molecular circuitry controlling virulence in *Francisella tularensis*. Genes Dev 31:1549–1560.

20. Travis BA, Ramsey KM, Prezioso SM, Tallo T, Wandzilak JM, Hsu A, Borgnia M, Bartesaghi A, Dove SL, Brennan RG, Schumacher MA. 2021. Structural Basis for Virulence Activation of *Francisella tularensis*. Mol Cell 81:139-152.e10.

21. Van Duin J, Wijnands R. 1981. The function of ribosomal protein S21 in protein synthesis. Eur J Biochem 118:615–9.

521 22. Chang C, Craven GR. 1977. Identification of several proteins involved in the messenger RNA binding
522 site of the 30 S ribosome by inactivation with 2-methoxy-5-nitrotropone. J Mol Biol 117:401–18.

523 23. Berk V, Zhang W, Pai RD, Cate JHD. 2006. Structural basis for mRNA and tRNA positioning on the
524 ribosome. Proc Natl Acad Sci U S A 103:15830–15834.

525 24. Watson ZL, Ward FR, Méheust R, Ad O, Schepartz A, Banfield JF, Cate JH. 2020. Structure of the
526 bacterial ribosome at 2 Å resolution. Elife 9:e60482.

527 25. Mizushima S, Nomura M. 1970. Assembly Mapping of 30S Ribosomal Proteins from *E. coli*. Nature
528 226:1214–1218.

529 26. Robertson WR, Dowsett SJ, Hardy SJS. 1977. Exchange of ribosomal proteins among the ribosomes
530 of *Escherichia coli*. Mol Gen Genet 157:205–214.

531 27. Lupski JR, Godson GN. 1984. The *rpsU-dnaG-rpoD* macromolecular synthesis operon of *E. coli*. Cell
532 39:251–252.

533 28. Deniziak M, Sauter C, Becker HD, Paulus CA, Giegé R, Kern D. 2007. Deinococcus glutaminyl-tRNA
534 synthetase is a chimer between proteins from an ancient and the modern pathways of aminoacyl-tRNA
535 formation. Nucleic Acids Res 35:1421–1431.

536 29. Searle BC, Swearingen KE, Barnes CA, Schmidt T, Gessulat S, Küster B, Wilhelm M. 2020. Generating
537 high quality libraries for DIA MS with empirically corrected peptide predictions. Nat Commun 11:1548.

538 30. Takata R. 1978. Genetic studies of the ribosomal proteins in *Escherichia coli* XI. Mol Gen Genet
539 160:151–155.

540 31. Nomura M, Gourse R, Baughman G. 1984. Regulation of the Synthesis of Ribosomes and Ribosomal
541 Components. Annu Rev Biochem 53:75–117.

542 32. Nano FE, Zhang N, Cowley SC, Klose KE, Cheung KKM, Roberts MJ, Ludu JS, Letendre GW,
543 Meierovics AI, Stephens G, Elkins KL. 2004. A *Francisella tularensis* Pathogenicity Island Required for
544 Intramacrophage Growth. J Bacteriol 186:6430–6436.

545 33. Larsson P, Oyston PCF, Chain P, Chu MC, Duffield M, Fuxelius H-H, Garcia E, Hälltorp G, Johansson
546 D, Isherwood KE, Karp PD, Larsson E, Liu Y, Michell S, Prior J, Prior R, Malfatti S, Sjöstedt A, Svensson K,
547 Thompson N, Vergez L, Wagg JK, Wren BW, Lindler LE, Andersson SGE, Forsman M, Titball RW. 2005.
548 The complete genome sequence of *Francisella tularensis*, the causative agent of tularemia. Nat Genet
549 37:153–159.

550 34. Nano FE, Schmerk C. 2007. The *Francisella* Pathogenicity Island. Ann N Y Acad Sci 1105:122–137.

551 35. Brotcke A, Weiss DS, Kim CC, Chain P, Malfatti S, Garcia E, Monack DM. 2006. Identification of
552 MglA-regulated genes reveals novel virulence factors in *Francisella tularensis*. Infect Immun 74:6642–
553 6655.

554 36. Yutin N, Puigbo P, Koonin EV, Wolf YI. 2012. Phylogenomics of prokaryotic ribosomal proteins. PLoS
555 One 7:e36972.

556 37. Galperin MY, Wolf YI, Garushyants SK, Alvarez RV, Koonin EV. 2021. Nonessential Ribosomal
557 Proteins in Bacteria and Archaea Identified Using Clusters of Orthologous Genes. J Bacteriol
558 203:e00058.

559 38. Bubunenko M, Baker T, Court DL. 2007. Essentiality of ribosomal and transcription antitermination
560 proteins analyzed by systematic gene replacement in *Escherichia coli*. J Bacteriol 189:2844–2853.

561 39. Yamamoto N, Nakahigashi K, Nakamichi T, Yoshino M, Takai Y, Touda Y, Furubayashi A, Kinjyo S,
562 Dose H, Hasegawa M, Datsenko KA, Nakayashiki T, Tomita M, Wanner BL, Mori H. 2009. Update on the
563 Keio collection of *Escherichia coli* single-gene deletion mutants. Mol Syst Biol 5:335.

564 40. Goodall ECA, Robinson A, Johnston IG, Jabbari S, Turner KA, Cunningham AF, Lund PA, Cole JA,
565 Henderson IR. 2018. The Essential Genome of *Escherichia coli* K-12. mBio 9:e02096-17.

566 41. Ramsey KM, Ledvina HE, Tresko TM, Wandzilak JM, Tower CA, Tallo T, Schramm CE, Peterson SB,
567 Skerrett SJ, Mougous JD, Dove SL. 2020. Tn-Seq reveals hidden complexity in the utilization of host-
568 derived glutathione in *Francisella tularensis*. PLoS Pathog 16:e1008566.

569 42. Jha V, Roy B, Jahagirdar D, McNutt ZA, Shatoff EA, Boleratz BL, Watkins DE, Bundschuh R, Basu K,
570 Ortega J, Fredrick K. 2020. Structural basis of sequestration of the anti-Shine-Dalgarno sequence in the
571 Bacteroidetes ribosome. Nucleic Acids Res 49:547–567.

572 43. Takada H, Morita M, Shiwa Y, Sugimoto R, Suzuki S, Kawamura F, Yoshikawa H. 2014. Cell motility
573 and biofilm formation in *Bacillus subtilis* are affected by the ribosomal proteins, S11 and S21. Biosci
574 Biotechnol Biochem 78:898–907.

575 44. Metselaar KI, Besten HMW den, Boekhorst J, Hijum SAFT van, Zwietering MH, Abee T. 2015.
576 Diversity of acid stress resistant variants of *Listeria monocytogenes* and the potential role of ribosomal
577 protein S21 encoded by *rpsU*. Front Microbiol 6:422.

578 45. Metselaar KI, Abee T, Zwietering MH, Besten HMW den. 2016. Modeling and Validation of the
579 Ecological Behavior of Wild-Type *Listeria monocytogenes* and Stress-Resistant Variants. Appl Environ
580 Microbiol 82:5389–5401.

581 46. Basco MDS, Kothari A, McKinzie PB, Revollo JR, Agnihothram S, Azevedo MP, Saccente M, Hart ME.
582 2019. Reduced vancomycin susceptibility and increased macrophage survival in *Staphylococcus aureus*
583 strains sequentially isolated from a bacteraemic patient during a short course of antibiotic therapy. J
584 Med Microbiol 68:848–859.

585 47. Blake KL, O'Neill AJ. 2013. Transposon library screening for identification of genetic loci
586 participating in intrinsic susceptibility and acquired resistance to antistaphylococcal agents. J
587 Antimicrob Chemother 68:12–16.

588 48. Friedman L, Alder JD, Silverman JA. 2006. Genetic Changes That Correlate with Reduced
589 Susceptibility to Daptomycin in *Staphylococcus aureus*. Antimicrob Agents Chemother 50:2137–2145.

590 49. Gutierrez MG, Yoder-Himes DR, Warawa JM. 2015. Comprehensive identification of virulence
591 factors required for respiratory melioidosis using Tn-seq mutagenesis. Front Cell Infect Microbiol 5:78.

592 50. Su J, Yang J, Zhao D, Kawula TH, Banas JA, Zhang J-R. 2007. Genome-Wide Identification of
593 *Francisella tularensis* Virulence Determinants. Infect Immun 75:3089–3101.

51. Mizuno CM, Guyomar C, Roux S, Lavigne R, Rodriguez-Valera F, Sullivan MB, Gillet R, Forterre P, Krupovic M. 2019. Numerous cultivated and uncultivated viruses encode ribosomal proteins. Nat Commun 10:752.

52. Al-Shayeb B, Sachdeva R, Chen L-X, Ward F, Munk P, Devoto A, Castelle CJ, Olm MR, Bouma-Gregson K, Amano Y, He C, Méheust R, Brooks B, Thomas A, Lavy A, Matheus-Carnevali P, Sun C, Goltsman DSA, Borton MA, Sharrar A, Jaffe AL, Nelson TC, Kantor R, Keren R, Lane KR, Farag IF, Lei S, Finstad K, Amundson R, Anantharaman K, Zhou J, Probst AJ, Power ME, Tringe SG, Li W-J, Wrighton K, Harrison S, Morowitz M, Relman DA, Doudna JA, Lehours A-C, Warren L, Cate JHD, Santini JM, Banfield JF. 2020. Clades of huge phages from across Earth's ecosystems. Nature 578:425–431.

53. Chen L-X, Jaffe AL, Borges AL, Penev PI, Nelson TC, Warren LA, Banfield JF. 2022. Phage-encoded ribosomal protein S21 expression is linked to late-stage phage replication. ISME Commun 2:31.

54. Maier TM, Havig A, Casey M, Nano FE, Frank DW, Zahrt TC. 2004. Construction and Characterization of a Highly Efficient *Francisella* Shuttle Plasmid. Appl Environ Microbiol 70:7511–7519.

55. Ramsey KM, Dove SL. 2016. A response regulator promotes *Francisella tularensis* intramacrophage growth by repressing an anti-virulence factor. Mol Microbiol 101:688–700.

56. Skeggs PA, Thompson J, Cundliffe E. 1985. Methylation of 16S ribosomal RNA and resistance to aminoglycoside antibiotics in clones of *Streptomyces lividans* carrying DNA from *Streptomyces tenjimariensis*. Mol Gen Genet 200:415–421.

612 57. Johnson GE, Li G-W. 2018. Genome-Wide Quantitation of Protein Synthesis Rates in Bacteria.
613 Methods Enzymol 612:225–249.

614

615 **FIGURES**
616

617 **Figure 1. *F. tularensis* ribosomes are heterogenous with respect to bS21. A.** Chart demonstrating
618 purity of wild-type ribosomes. Categories represent classification of proteins identified by mass
619 spectrometry of ribosomes purified from wild-type *F. tularensis* LVS cells. Numbers represent the
620 percentage of spectral counts corresponding to proteins in each category, combined from
621 quadruplicate samples. **B.** Wild-type *F. tularensis* LVS ribosomes contain more than one bS21 homolog.
622 Table detailing the number of spectral counts corresponding to bS21 homologs identified from
623 individual ribosome purifications (A – D) from wild-type cells. Spectral counts corresponding to bS21-1
624 and/or bS21-3 cannot be unambiguously assigned due to complete sequence identity of detected
625 peptides. ND: not detected. **C.** Each bS21 homolog can be incorporated into ribosomes. Top: Sucrose
626 gradient sedimentation profile from actively-translating wild-type cells containing an empty vector.
627 Nucleic acid content was monitored by A260 (y-axis). Peaks corresponding to the 30S, 50S, 70S, and
628 polysomes are indicated. Fractions collected are indicated on the x-axis. Bottom: Immunoblot analysis
629 of fractions from sucrose gradient sedimentation performed on actively-translating cells ectopically
630 expressing indicated bS21 homolog with VSV-G epitope tag. Wells correspond to fractions 1 – 21 from
631 profile above.
632

633 **Figure 2. Loss of bS21-2 leads to changes in protein abundance that cannot be explained by changes**
634 **in transcript abundance.** Cells with (WT, wild-type) and without bS21-2 (Δ*rpsU2*) were analyzed using
635 RNA-Seq (x-axis) and DIA whole cell mass spectrometry (y-axis). Genes are represented by dots. Most
636 genes with changes in protein (161 yellow dots) do not have corresponding changes in transcript
637 abundance. One gene (orange dot) has discordant changes in transcript and protein abundance. Green

dots (23) represent genes with concordant changes in transcript and protein abundance. Blue dots (60) indicate genes with altered transcript abundance only. Horizontal dashed lines indicate +/- 1.5-fold cutoff for differential protein abundance; vertical dashes indicate +/- 2-fold cutoff for differential transcript abundance. Colored dots with black outlines represent genes with significant changes in protein (+/- 1.5-fold change, adjusted p-value <0.05) and/or transcript (+/- 2-fold change, adjusted p-value <0.05) abundance as indicated above, while grey dots without outline represent genes with changes that did not meet the statistical thresholds. Three grey dots are located outside the bounds of the axis as represented.

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

Figure 4. Cells without bS21-2 have an intramacrophage growth defect, which can be complemented by ectopic expression of bS21-2. Growth and survival of F. tularensis LVS cells within J774A.1 cells.

660 Murine macrophage-like J774A.1 cells were infected with indicated bacterial cells at a multiplicity of
661 infection of 5 – 10. J774A.1 cells were lysed and bacteria were plated for enumeration (colony forming
662 units [CFU]) at 2 and 24 hours post-infection. Error bars represent 1 SD. Experiments were repeated at
663 least twice and data from a representative experiment are shown. Lines above bars indicate statistical
664 comparison among groups by t-test. Asterisk indicates group to which all other groups are compared, if
665 horizontal line connects to line above group, $*p < 0.05$ using Benjamini-Hochberg correction.
666
667 **SUPPLEMENTAL MATERIAL**
668
669 **SUPPLEMENTAL FIGURES**
670
671 **Figure S1. *F. tularensis* encodes three *rpsU* genes.** *F. tularensis rpsU2*, which encodes bS21-2, is
672 syntenic with the only *rpsU* in *E. coli*, which is located in the macromolecular synthesis operon (1). This
673 operon in *E. coli* includes *rpsU* (encoding bS21), *dnaG* (encoding DNA primase), and *rpoD* (encoding
674 RNA polymerase σ^{70}). In *F. tularensis*, this operon also includes *yqeY*, the product of which may be
675 involved in tRNA aminoacylation. *rpsU1*, encoding bS21-1, is located immediately downstream of *cspC*
676 (encoding cold-shock protein CspC), while *rpsU3*, encoding bS21-3, is not apparently in an operon with
677 other genes. Genomic locations of *rpsU* genes were determined using RefSeq NC_007880 for *F.*
678 *tularensis* and NC_000913 for *E. coli*.
679
680 **Figure S2. The three bS21 homologs in *F. tularensis* are distinct.** Percent identities of amino acid
681 sequences for *F. tularensis* LVS bS21-1, bS21-2, bS21-3 and *E. coli* bS21 were calculated using the

682 multiple sequence alignment tool ClustalOmega (2). The bS21 homologs in *F. tularensis* are similar to
683 each other, particularly bS21-1 and bS21-3 which are 72% identical at the amino acid level. bS21-2,
684 encoded by the *rpsU* homolog gene syntenic to the single *E. coli rpsU* gene, is also the most similar to
685 *E. coli* bS21, with 60% amino acid identity.
686
687 **Figure S3. Each bS21 homolog can be detected in translationally-active ribosomes.** For **A – D**, top:
688 Sucrose gradient sedimentation profile from actively-translating wild-type *F. tularensis* cells with either
689 empty vector or ectopic expression of indicated bS21 homolog. Nucleic acid content was monitored by
690 A260 (y-axis). Peaks corresponding to the 30S, 50S, 70S, and polysomes are indicated. Fractions
691 collected are indicated on the x-axis. For **A – D**, bottom: Immunoblot analysis of fractions from sucrose
692 gradient sedimentation (above), probing for VSV-G. Wells correspond to fractions 1 – 21 from profile
693 above. **A.** Cells from wild-type *F. tularensis* LVS with empty vector (LVS pF). **B.** Cells from wild-type *F.*
694 *tularensis* LVS with ectopic expression of bS21-1 (LVS pF-bS21-1-V). **C.** Cells from wild-type *F. tularensis*
695 LVS with ectopic expression of bS21-2 (LVS pF-bS21-2-V). **D.** Cells from wild-type *F. tularensis* LVS with
696 ectopic expression of bS21-3 (LVS pF-bS21-3-V).
697
698 **Figure S4. Loss of bS21-2 does not affect transcript abundance of FPI-encoded genes.** Quantitative
699 real-time PCR was used to determine the relative transcript abundance for indicated FPI genes in wild-
700 type cells, cells lacking bS21-2 (Δ*rpsU2*), or cells lacking the transcription factor PigR (Δ*pigR*). Cells
701 lacking PigR serve as a positive control, as PigR positively regulates its own transcription and the
702 transcription of *pdpA*, *pdpB*, and *iglA*. The *rpoA1* and *bfr* genes are included as negative controls, as
703 their expression is not influenced by bS21-2 or PigR. Transcript abundances are normalized to *tul4*,

whose expression is not influenced by bS21-2 or PigR. Error bars represent 1 SD from the value (calculated using the mean threshold cycle). ns: not significant. ND: not detected *adjusted $p < 0.05$ by t-test.

Figure S5. Loss of bS21-2 does not affect protein degradation of PdpB. One-phase decay of PdpB from antibiotic-chase experiment from wild-type cells and cells lacking bS21-2 (Δ*rpsU2*). Neither strain showed significant degradation of PdpB through the time points assessed; the calculated half-life for both was greater than 120 minutes. Y-axis is logarithmic and error bars represent 1 standard deviation from the mean.

SUPPLEMENTAL TABLES

Table S1. Proteins associated with purified *F. tularensis* ribosomes. LC-MS/MS analysis of four samples of ribosomes purified from wild-type cells by sucrose cushions. Total spectral counts (columns F-I) were filtered with the following parameters: 99% protein threshold, 95% peptide threshold, minimum of 2 peptides. Hypothetical proteins with no known function were not categorized as transcription or translation-related. Proteins were primarily ribosomal (69%) or associated with transcription and translation processes (10%).

Table S2. Cells lacking bS21-2 exhibit genome-wide changes in protein abundance. Data-independent acquisition (DIA) mass spectrometry analysis of cellular lysates was used to quantify genome-wide protein abundance in wild-type cells (WT), cells lacking bS21-1 (delt_rpsU1), cells lacking bS21-2

(delt_rpsU2), and cells lacking bS21-3 (delt_rpsU3). Each deletion strain was compared to wild-type, but significant changes (>1.5-fold change, adjusted p-value <0.05, excluding bS21) were only observed in the cells lacking bS21-2. Cells are highlighted if the fold-change (columns R, U, and X) is greater than 1.5 (log_2FC>0.58 or <-0.58). Green indicates less abundant in deletion strains compared to wild-type, and red indicates more abundant. Adjusted p-values are highlighted red if <0.05 (columns T, W, and Z).

Table S3. Cells lacking bS21-2 have significant changes in transcript abundance. RNA-Seq was used to compare genome-wide transcript abundance from wild-type cells with an empty vector (LVS pF), cells lacking bS21-2 and containing an empty vector (LVS Δ*rpsU2* pF), and bS21-2 mutant cells with bS21-2-V ectopically expressed (LVS Δ*rpsU2* pF-*rpsU2*-V). Transcripts with significant differences in cells lacking bS21-2 compared to wild-type (>2-fold change, adjusted p-value <0.05) are included (columns E-F). All changes were complemented by ectopic expression of bS21-2-V (columns G-J). Base mean (column D) reflects a measure of transcript abundance across all strains.

Table S4. RNA-Seq reveals that cells without bS21-2 do not have transcript reductions across the Francisella Pathogenicity Island. Of the 16 Francisella Pathogenicity Island (FPI) genes encoded by *F. tularensis* LVS, only two genes are significantly differentially expressed at the transcript level in cells lacking bS21-2 compared to wild-type (log_2FC>1.00 or <-1.00, adjusted p-value <0.05). These changes are complemented by ectopic expression of bS21-2-V on a plasmid.

Table S5. Comparison of *in vitro* and intramacrophage growth rates for strains used in this study. *In vitro* growth was assessed during early exponential phase by measuring OD_{600}. *In vitro* generation

748 times for LVS pF, LVS Δ*rpsU2* pF, and LVS Δ*rpsU2* pF-bS21-2-V were calculated from three independent
749 experiments, others were calculated from two. Generation times for intramacrophage growth are
750 averages across three independent experiments and were determined by comparison of CFU
751 recovered after 2 versus 24 hours. +/- values indicate SD.
752
753 **Supplemental References**
754

755 1. Lupski JR, Godson GN. 1984. The *rpsU-dnaG-rpoD* macromolecular synthesis operon of *E. coli*. Cell
756 39:251–252.

757 2. Madeira F, Pearce M, Tivey ARN, Basutkar P, Lee J, Edbali O, Madhusoodanan N, Kolesnikov A, Lopez
758 R. 2022. Search and sequence analysis tools services from EMBL-EBI in 2022. Nucleic Acids Res
759 50:W276–W279.

760