

**Ribosome heterogeneity results in leader sequence-mediated
regulation of protein synthesis in *Francisella tularensis***

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Abstract

While the impacts of ribosomes are generally examined in bulk, there is accumulating evidence that heterogeneity in ribosome composition may lead to altered function and provide ribosomes the capacity to regulate protein synthesis. Ribosome heterogeneity in *F. tularensis* results from incorporation of one of three homologs of bS21, a small ribosomal protein demonstrated to regulate protein synthesis in other bacteria. Loss of one homolog, bS21-2, results in genome-wide post-transcriptional changes in protein abundance. This suggests that bS21-2 can, either directly or indirectly, lead to preferential translation of particular mRNAs. In this study, we examine the potential for bS21-2 to function indirectly (via Hfq) and in a leader sequence-dependent manner. We found that loss of bS21-2 leads to increased abundance of the RNA chaperone Hfq and both Hfq and bS21-2 impact expression of key virulence genes, but these two proteins influence protein abundance via distinct mechanisms. In contrast, the 5' untranslated region (UTR) of some bS21-2 responsive genes, including key virulence genes, is sufficient to lead to changes in translation in cells lacking bS21-2. Focusing on a particular 5' UTR, we identified key elements critical for responsiveness to bS21-2. Specifically, we found that the 5' UTR must have an imperfect Shine-Dalgarno sequence and, in at least one 5' UTR, a specific 6-nucleotide sequence for bS21-2-responsive translation. Our results are consistent with a model in which a bS21 homolog improves translation initiation through interactions with specific leader sequences. Together, we determined that ribosome composition in *F. tularensis* regulates

translation in a leader sequence-dependent manner, a finding which may extend to many other bacteria.

Importance

Ribosome heterogeneity is common in bacteria and there is mounting evidence that ribosome composition plays a regulatory role in protein synthesis. However, mechanisms of ribosome-driven gene regulation are not well understood. In the human pathogen *Francisella tularensis*, which encodes multiple homologs for one ribosomal protein, bS21, loss of one homolog impacts protein synthesis and virulence. Here, we explore the mechanism behind the changes in protein synthesis and find that the ribosomal protein bS21-2 does not function coordinately with the RNA chaperone Hfq. Rather, changes in protein synthesis can be linked to specific sequences in the leader of transcripts in the absence of strong mRNA-ribosome interactions. Our data support a model in which ribosome composition regulates gene expression through translation, a strategy that may be conserved in diverse organisms with various source of ribosome heterogeneity.

Introduction

Ribosomes, the molecular machines that synthesize proteins, can be heterogeneous in structure (Genuth & Barna, 2018). As bacterial ribosomes are composed of 3 ribosomal RNA molecules (rRNAs) and ~ 50 ribosomal proteins (r-proteins), heterogeneity can arise from differences in rRNA sequence among *rrn* operons, rRNA posttranscriptional modification, r-protein content, or r-protein posttranslational modification (Byrgazov et al., 2013). The consequences of ribosome heterogeneity are incompletely understood, and much debate surrounds the hypothesis that distinct classes of ribosomes can have specialized functions by preferentially translating subsets of mRNA (Ferretti & Karbstein, 2019). The 30S subunit r-protein bS21 is one of the last assembled proteins in the ribosome and is easily exchanged among ribosomes (Mizushima & Nomura, 1970; Robertson et al., 1977), allowing bS21 to be a source of ribosomal heterogeneity.

In *E. coli*, bS21 is involved in translation initiation (van Duin & Wijnands, 1981; Chang & Craven, 1977), and multiple studies have suggested that bS21 might play a regulatory role in gene expression (Mizuno et al., 2019; Jha et al., 2020; Chen et al., 2022; Trautmann & Ramsey, 2022). Recent work in the Bacteroidia species *Flavobacterium johnsoniae* clearly demonstrated that bS21 controls gene expression. In this organism, incorporation of bS21 into the ribosome contributes to sequestration of the anti-Shine Dalgarno (ASD) (Jha et al., 2020). The mRNA encoding bS21, *rpsU*, is one of the only *F. johnsoniae* mRNAs with a strong Shine-Dalgarno (SD) sequence. Depletion of bS21 or removal of the region of bS21 necessary for ASD sequestration results in increased translation from the

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

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

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

lineages, including the Gammaproteobacteria. Thus, in the current study we aim to understand the mechanisms by which bS21-2 affects translation in *F. tularensis*.

Using qPCR and immunoblot analyses, we show that bS21-2 and the RNA-binding protein Hfq both influence T6SS proteins, but do not act in a coordinated manner and function via distinct pathways. Through reporter assays using translational fusions, we see that the 5' untranslated region (5' UTR) of some transcripts is sufficient to cause differences in protein production if bS21-2 is lost, indicating that these 5' UTRs are responsive to bS21-2. By mutagenizing the sequences of bS21-2-responsive 5' UTRs, we also found that transcripts with ideal SD sequences do not require bS21-2 for efficient translation. In an attempt to identify which component of the 5' UTR is driving the responsiveness to bS21-2, we concluded that the secondary structure of the leader sequence does not play a clear role and two STREME-identified motifs that are enriched in bS21-2-impacted genes are not needed to cause changes in protein abundance. Finally, we identified a short nucleotide sequence in the 5' UTR of *mraY* that is critical for bS21-2-mediated translation. Our findings reveal that an r-protein homolog, bS21-2, governs protein abundance by influencing translation from mRNA species with specific leader sequences.

Results

bS21-2 and Hfq influence T6SS protein abundance via different mechanisms

While the small subunit ribosomal protein bS21-2 post-transcriptionally governs the abundance of many genes, including those that encode T6SS proteins, the mechanism by which this occurs is unknown (Trautmann & Ramsey, 2022). In considering the control of the T6SS proteins, we first hypothesized that bS21-2 may be exerting its effects indirectly, by modifying the abundance of a regulator that directly controls production of the T6SS proteins. The regulatory RNA-binding protein Hfq is known to control gene expression post-transcriptionally in many organisms and has been shown to impact the expression of T6SS proteins in *F. tularensis* (Meibom et al., 2009; Lenco et al., 2014). Our proteomics analysis identified increased Hfq in cells lacking bS21-2 compared to wild-type (5.9-fold), although substantial variation in biological replicates precluded the differences from reaching statistical significance (adj p=0.066; Trautmann & Ramsey, 2022). Additionally, many of the genes impacted by bS21-2 have AU-rich 5' UTRs that resemble ARN-motifs, known targets of Hfq (Link et al., 2009). This led us to hypothesize that Hfq may play a role in bS21-2-mediated regulation of T6SS proteins.

To validate the previous proteomics data and confirm that cells lacking bS21-2 do have increased Hfq, we added DNA specifying a C-terminal vesicular stomatitis virus glycoprotein (VSV-G) epitope tag to *hfq* in cells with (WT) and without ($\Delta rpsU2$) bS21-2 and determined relative protein abundance by

quantitative immunoblotting. We found a moderate increase in Hfq (about 30%, **Fig 1A**) in cells lacking bS21-2 compared to wild-type; this is consistent with our proteomics findings, including that it is not a difference that would have reached our significance threshold.

Yet given the detectable increase in Hfq in cells lacking bS21-2, we sought to determine if this is due to increased translation of the *hfq* mRNA. Using a translational fusion in a GFP reporter assay (described in more detail below), we assessed the relative translation of mRNAs containing the *hfq* 5' UTR. Briefly, reporters expressing either the 5' UTR of *hfq* or a control 5' UTR (*tul4*) fused to *gfp* were introduced into cells with and without bS21-2. We found that there is approximately 1.88-fold more GFP produced from the 5' UTR of *hfq* in cells lacking bS21-2, indicating this UTR leads to more efficient translation by ribosomes without bS21-2 (**Fig 1B**). This suggests that the observed increase in Hfq in cells lacking bS21-2 is likely due to increased translation initiation.

If the moderate increase in Hfq leads to the observed reduction in T6SS proteins when bS21-2 is absent, Hfq must be acting as a repressor of the T6SS. However, inconsistent results have been reported with respect to the role of *F. tularensis* Hfq in regulating the T6SS genes. A transcriptomic analysis of cells lacking Hfq found that one of the two Francisella Pathogenicity Island (FPI) operons encoding the T6SS, the *pdpA* operon, was upregulated in *hfq* mutant cells (Meibom et al., 2009). A proteomics study of *hfq* mutant cells determined that proteins encoded by the other FPI operon, the *iglA* operon, are less abundant and

found no change in the abundance of proteins encoded by the *pdpA* operon (Lenco et al., 2014). To clarify the impact of Hfq on T6SS protein abundance, we determined the abundance of several T6SS proteins encoded on both FPI operons in cells with and without Hfq by quantitative immunoblotting (**Fig 1C**) (Trautmann & Ramsey, 2022). PdpB, encoded in the *pdpA* operon, was more abundant in cells without Hfq compared to wild-type (>2 -fold; $p<0.01$), while IglB and IglA, encoded on the *iglA* operon, were not impacted by the loss of Hfq (**Fig 1C**). This is in contrast to cells lacking bS21-2, which contained reduced amounts of all three proteins (**Fig 1C**; Trautmann & Ramsey, 2022). These data suggest that Hfq regulates expression of proteins encoded by the *pdpA* operon, but not the *iglA* operon, consistent with Meibom et al. (2009).

Hfq can exert its effects through a variety of mechanisms, some of which result in changes in translation initiation. To determine if the presence of *F. tularensis* Hfq impacts the translation of T6SS proteins (like bS21-2), we analyzed the ability of cells lacking Hfq to translate mRNAs containing either the 5' UTRs of the T6SS protein gene *pdpA* or control gene *tul4* fused to *gfp*. We found that translation of mRNAs with the *pdpA* or *tul4* 5' UTRs are not altered when Hfq is absent compared to wild-type, while *pdpA* translation decreases if bS21-2 is absent (**Fig 1D**). Thus, Hfq-associated changes in T6SS proteins are not due to changes in translation initiation.

As work from the Charbit lab indicates that Hfq represses *pdpA* operon transcript abundance (Meibom et al., 2009) and we found that Hfq does not impact

translation of the *pdpA* 5' UTR, we hypothesized that cells lacking Hfq might have increased PdpB due to increased *pdpB* (and *pdpA* operon) transcript abundance. We compared mRNA isolated from wild-type cells as well as those lacking bS21-2 ($\Delta rpsU2$) or Hfq (Δhfq) cells by qPCR, and found that *pdpA* and *pdpB* transcripts have large, statistically significant increases when Hfq is not present (42-fold and 69-fold, respectively) but the relative impact on *iglA* transcript is minor (2.5-fold increased) (**Fig 1E**); neither *pdpA*, *pdpB*, nor *iglA* transcripts are meaningfully impacted by the loss of bS21-2.

If bS21-2 influences the abundance of T6SS proteins indirectly, through another post-transcriptional regulator, we expect that regulator would influence translation of essentially all the T6SS proteins without impacting transcript abundance. We have demonstrated that bS21-2 represses Hfq production and Hfq functions as a negative regulator of T6SS protein; this network could in theory lead to increased T6SS proteins in cells lacking bS21-2. But critically, Hfq only represses T6SS proteins encoded on the *pdpA* operon, and does so by reducing transcript abundance. Thus, our results suggest two distinct pathways of regulation for the genes encoding the T6SS: one in which bS21-2 improves efficiency of translation initiation from both operons, and one in which Hfq represses transcript abundance of only the *pdpA* operon.

bS21-2 promotes translation of specific genes in a 5' UTR-dependent manner

In *E. coli*, bS21 has been implicated in sequence-dependent translation initiation and is located adjacent to the 5' untranslated region (UTR) of mRNAs

during translation initiation (Kaledhonkar et al., 2019; van Duin & Wijnands, 1981). Loss of bS21-2 in *F. tularensis* leads to changes in abundance for a subset of the proteome (Trautmann & Ramsey, 2022). This led us to hypothesize that bS21-2 may directly impact protein abundance by modulating translation initiation in a 5' UTR-dependent manner. In order to test this hypothesis, our goal was to assess the role of 5' UTR sequences in bS21-2-mediated translation of particular genes. Thus, we developed a series of reporter constructs that consisted of the experimentally determined or predicted 5' UTR with the first 6 codons of the gene of interest, fused to a reporter gene (*lacZ* or *gfp*) (**Fig 2a**; also as in **Fig 1B** and **1D**). Reporter constructs were expressed by the *tul4* promoter, which is unaffected by the presence or absence of bS21-2 (Trautmann & Ramsey, 2022). This design allows for comparable transcription of reporter genes in both genotypes so that we can compare relative translation initiation. The reporter constructs were introduced into *F. tularensis* cells with (WT) or without bS21-2 ($\Delta rpsU2$). Some experiments were completed using β -galactosidase reporters incorporated into the chromosome at the Tn7 site. Toxicity of plasmids that produce high levels of β -galactosidase in *E. coli* during plasmid production led us to use a GFP-based reporter system for some constructs. Reporter constructs using *gfp* were cloned into a multi-copy plasmid that is retained at essentially the same copy number in *F. tularensis* cells with and without bS21-2 (**Fig S1**).

These reporter assays evaluated the relative efficiency of translation initiation of specific 5' UTRs in cells with or without bS21-2. We chose to assess the 5' UTRs corresponding to proteins with significant changes only in protein

abundance in cells lacking bS21-2 (Trautmann & Ramsey, 2022). Consistent with the observed changes in protein abundance being due to changes in translation initiation, we found that the 5' UTRs of *pdpA*, *iglA*, *mraY*, FTL_0222, or FTL_1093 genes fused to GFP led to significantly less fluorescence in cells lacking bS21-2 compared to wild-type (**Fig 2B**). In contrast, the 5' UTR of *tul4*, a gene not differentially expressed in cells lacking bS21-2, did not lead to a significant decrease in fluorescence in cells lacking bS21-2 (**Fig 2B**). These data reveal that the 5' UTR of a gene is sufficient for bS21-2 to affect translation and is consistent with the idea that bS21 may be regulating translation initiation. We will refer to 5' UTRs that result in altered protein abundance in the presence of bS21-2 as “bS21-2-responsive.” We also found that the 5' UTRs of some genes impacted by bS21-2 in our proteomics analysis did not lead to reporter gene differences in cells lacking bS21-2, including FTL_0881 and FTL_0215 (**Fig S2**). We do not have experimentally determined transcription start sites for these genes, so it is possible the lack of regulation is due to inaccurate 5' UTR predictions. Other possibilities, including indirect regulation of these particular genes, are described in the discussion.

An ideal Shine-Dalgarno sequence masks the positive effects of bS21-2 on translation

Given that the FPI gene *pdpA* has a bS21-2-responsive 5' UTR (**Fig 1D and 2A**), we further examined features of the *pdpA* 5' UTR that may lead to preferential translation from ribosomes containing bS21-2. Based on structures of

the *E. coli* ribosome during translation initiation, the bS21 residue R17 is close enough to directly contact the 16S rRNA nucleotide C1529, which is part of the anti-Shine Dalgarno (ASD) sequence (Kaledhonkar et al., 2019; **Fig 3A**). R17 is conserved in all three *F. tularensis* bS21 homologs and the rRNA-encoded ASD is identical in *F. tularensis* and *E. coli*. Thus, we hypothesized that bS21 homologs in *F. tularensis* may also contact the ASD and influence Shine-Dalgarno (SD)-ASD binding during translation initiation. To test this possibility, we developed β -galactosidase translational reporters with altered SD sequences in the *pdpA* 5' UTR (**Fig 3B**). 5' UTRs with mutations that retained imperfect base-pairing between the ASD and SD (*badSD*, *tul4SD*) were still bS21-2-responsive. However, introducing an ideal SD, in two different positions (*idealSD*, *ideal_movedSD*), led to similar reporter gene expression in cells with and without bS21-2, indicating that these 5' UTRs are no longer bS21-2-responsive (**Fig 3B**).

We replicated the impact of a perfect SD on the bS21-2 responsive 5' UTR of another gene, *mraY*. Modification of the imperfect *mraY* SD to an ideal SD resulted in no significant difference in GFP production in cells with or without bS21-2 (**Fig 3C**). It is worth noting that in each of these cases, the addition of a perfect SD in the correct location (separated from the start codon by 6 to 8 nt) leads to increased total reporter production (**Fig S3**). These data suggest that genes with perfect SD sequences do not require bS21-2 for efficient translation; in other words, an ideal SD may lead to such efficient translation that any response to bS21-2 becomes negligible.

Because perfect SD sequences mask the impacts of bS21-2, we hypothesized that genes with weaker SD sequences may require bS21-2 for efficient translation. We compared predicted SDs for genes whose proteins are positively affected (n=74), negatively affected (n=84), or unaffected (n=82) by bS21-2 (Trautmann & Ramsey, 2022). We found that the genes positively affected generally have weaker SD sequences, with only 39% having strong SD sequences (defined by 4 or more nucleotides [nt] complementary to the ASD), compared to 54% or 69% strong SDs in negatively affected or unaffected genes, respectively (**Fig 3D**). These data are consistent with a model in which bS21-2 influences translation initiation predominately in the absence of strong SD-ASD interactions. However, given that many genes have weak or non-perfect SDs and are not affected by bS21-2, there is an unidentified component of the 5' UTR that results in responsiveness to bS21-2.

Sequence-specific motifs found in the 5' UTR of genes governed by bS21-2 do not alter bS21-2 responsiveness

We reasoned that 5' UTRs may be responsive to bS21-2 because they harbor a common sequence-specific element that may mediate an altered interaction with bS21-2 or bS21-2-containing ribosomes. To identify such an element, we compiled 5' UTR sequences including 100 nts upstream of the start codon and the first six codons of the gene for all proteins that were significantly less abundant in cells lacking bS21-2 compared to wild-type (n=74; 100 nt was arbitrarily chosen because most *F. tularensis* transcription start sites have not been

identified). As a control, we also compiled 5' UTR sequences from 82 genes that were not impacted by bS21-2 presence. Using the motif-finding algorithm STREME, which identifies ungapped motifs enriched in large data sets (Bailey, 2021), we identified sequence motifs enriched in the 5' UTRs of the 20 genes most positively governed by bS21-2 (Trautmann & Ramsey, 2022). The motifs we identified were enriched compared to shuffled sequences and were not found to be enriched in the control sequences. Two motifs, which we refer to as Motif 1 and Motif 2, are AU-rich and are found in 19 and 18 of the 20 sequences assessed, respectively (**Fig 4A**). Targeted mutations were made to modify these motifs in the *mraY* 5' UTR and assess their impact on responsiveness to bS21-2. Mutations 1 and 2 modified Motif 1 from AAAUAAC to CCCGCCG, which altered the AU-content of the entire motif, and AAUAUACA, which altered the three most conserved nt in the motif. When assessed using the GFP reporter assay, neither of these modifications altered the responsiveness of the 5' UTR to bS21-2 (**Fig 4B**). To assess the contribution of Motif 2, we created mutation 5, a truncation of the 5' end of the *mraY* 5' UTR that removed 25 nt including motif 2. Similarly, this 5' UTR also remained responsive to bS21-2 (**Fig 6**). Together, these data indicate that neither of the two STREME-predicted AU-rich motifs are necessary for the positive impact of bS21-2 on translation of the *mraY* 5' UTR.

Predicted secondary structures of 5' UTRs are not responsible for bS21-2 responsiveness

The secondary structure of mRNA molecules is an important determinant of translation initiation efficiency (Hall et al., 1982; de Smit & van Duin, 1994). We hypothesized that the secondary structure of mRNAs may play a role in bS21-2-mediated translation. We predicted the secondary structure of the *pdpA* 5' UTR using MXfold2 (**Fig S4**), made targeted mutations to disrupt the predicted stem-loop structure (*pdpA* mut1), and generated β -galactosidase reporters at the Tn7 site in cells with (WT) and without ($\Delta rpsU2$) bS21-2 (**Fig 5A**). We also made complementary mutations to restore the original predicted secondary structure without maintaining the original sequence (*pdpA* mut2). In designing each mutation, we ensured that there was no significant disruption to the Shine-Dalgarno or start codon. Neither of these variants that altered the *pdpA* 5' UTR structure affected responsiveness to bS21-2, indicating that the secondary structure of this 5' UTR does not play a role in translation modulation by bS21-2.

We then looked at the secondary structure of a longer 5' UTR, *mraY*, which was predicted using MXFold2 to contain a large stem-loop (**Fig 5B**). We mutated a region that was predicted to form the stem closest to the loop, thereby disrupting the structure (*mraY* mut3). We also made complementary mutations to restore the structure (*mraY* mut4) and assessed these 5' UTRs in a GFP reporter assay. We found that the disruption to the predicted secondary structure (*mraY* mut3) did not affect bS21-2 responsiveness. In contrast, the complementary mutation that

restored the stem-loop structure was no longer responsive to bS21-2 (**Fig 5B**). In our studies of two different bS21-2 responsive 5' UTRs, we did not find a specific secondary structure that is necessary for bS21-2-responsive translation.

A 6-nucleotide region of the *mraY* 5' UTR is critical for bS21-2-responsiveness

While testing the importance of the *mraY* 5' UTR structure to bS21-2 responsiveness, we identified a 5' UTR variant that was no longer responsive to bS21-2 (*mraY* mut4). This variant included mutations in nt 58-63 upstream of the initiation codon. To further clarify the sequence necessary for bS21-2-responsiveness in the *mraY* 5' UTR, we made a series of truncations and modifications from the 5' end of the leader sequence. Truncating the 5' UTR to 75 nt (*mraY* mut5) did not impact bS21-2-responsiveness, nor did modifying the AU-rich region located 64-70 nt from the initiation codon (*mraY* mut8) (**Fig 6**). But truncating the 5' UTR to 57 nt (*mraY* mut6) led to loss of bS21-2-responsiveness, further implicating the nt 58-63 upstream of the initiation codon, GACUCU, in responsiveness to bS21-2, as suggested by *mraY* mut4 (**Fig 5B**). We further assessed the importance of the GACUCU sequence using *mraY* mut7 (truncating the 5' UTR to 60 nt and changing the first three nt to AGA) and *mraY* mut9 (truncating the 5' UTR to 63 and mutating nt 58-63 to AGUGAG), and found neither was responsive to bS21-2. These data allow us to conclude that the nt 58-63 upstream of the *mraY* initiation codon, GACUCU, are critical for bS21-2-responsive translation of the *mraY* 5' UTR. This is consistent with a model in which bS21-2-

containing ribosomes interact directly or indirectly with a specific element of the leader sequence to facilitate efficient translation initiation of some transcripts.

Discussion

In this work, we addressed two hypotheses regarding how bS21-2 exerts its effects on protein synthesis. In the first, we suggested that the effects of bS21-2 on the T6SS proteins may be mediated by Hfq, a known regulator of T6SS proteins. However, since there have been conflicting reports regarding the impacts of Hfq on the T6SS, we also examined the effects of Hfq on T6SS protein and transcript abundance. Our work clearly demonstrates that Hfq is a negative regulator of one of the two FPI operons encoding T6SS proteins and that this regulation influences transcript abundance rather than translation, consistent with and building upon, a prior study (Meibom et al., 2009). In contrast, the positive effects of bS21-2 on essentially all T6SS proteins can be attributed to differences in protein synthesis. Thus, we conclude that Hfq and bS21-2 function in independent pathways to regulate the T6SS proteins. The second hypothesis we addressed is that ribosomes containing bS21-2 may influence translation initiation of specific transcripts in a leader sequence-dependent manner. Using reporter assays, we determined that specific 5' UTR sequences are sufficient to lead to altered translation in cells with or without bS21-2. In a comprehensive assessment of 5' UTR elements, we found that bS21-2 responsive 5' UTRs have imperfect SD sequences and, in a specific responsive 5' UTR, the presence of a particular six

nucleotide sequence. Together, these results suggest that bS21-2 impacts protein synthesis by altering translation initiation on mRNAs with specific leader sequences.

In *F. tularensis*, loss of the RNA chaperone Hfq results in defective intramacrophage replication, which is essential for virulence. Yet how Hfq promotes *F. tularensis* intramacrophage replication remains poorly-understood. Few small RNAs have been identified in *F. tularensis* and none have been identified that are Hfq-dependent (Postic et al., 2010; Postic et al., 2012). Two prior studies identified that cells without Hfq have altered expression of the T6SS genes, encoded on the FPI (Meibom et al., 2009; Lenco et al., 2014). One study reported increased transcript abundance of only the *pdpA* operon genes, while the other identified decreased abundance of proteins encoded by the *iglA* operon (Meibom et al., 2009; Lenco et al., 2014). Our results corroborate the former report and we additionally demonstrate a concordant increase in protein abundance for a *pdpA*-operon encoded gene. Finally, using a translational reporter fusion, we show that changes in protein abundance for genes in the *pdpA* operon are not due to changes in translation efficiency. These results demonstrate that Hfq acts to repress *pdpA* operon transcript abundance but does not appear to influence T6SS protein synthesis. The molecular mechanism by which Hfq exerts its effects on this operon, and if it involves a small RNA, remain unclear. Regardless, the change in production of T6SS components is consistent with the observed intramacrophage growth defect, as *F. tularensis* cells overproducing the T6SS are defective for intramacrophage survival (Rohlfing et al., 2018).

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

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

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

is notable that this 6 nt sequence, which is found 58-63 nt away from the initiation codon, is predicted to form a stem-loop complementary to sequence 3 nt away from the SD. Yet disruption of that structure by mutating the complementary sequence does not impact the response to bS21-2, implicating the 6 nt sequence alone. Further work will be necessary to determine if this sequence is sufficient for bS21-2 responsiveness, to identify the commonalities among bS21-2 responsive 5' UTRs, and to determine how bS21-2 influences translation initiation on specific leader sequences.

Materials and Methods

Bacterial strains and growth conditions

Unless otherwise noted, bacterial strains were grown as indicated. *Francisella tularensis* subsp. *holarctica* Live Vaccine Strain (LVS) cells were grown in Mueller-Hinton broth (BD Difco) supplemented with 0.025% iron pyrophosphate, 0.1% glucose, and 2% Isovitalex (sMHB), shaking aerobically or on cystine heart agar (BD Difco or prepared in house) plates with 1% hemoglobin (CHA-H) at 37°C. *Escherichia coli* XL1-Blue, DH5 α (New England Biolabs), and DH5 α λ -pir cells were grown in lysogeny broth (LB) shaking aerobically or on LB agar plates at 37°C. Kanamycin was used at concentrations of 5 μ g/mL (*F. tularensis*) or 50 μ g/mL (*E. coli*); hygromycin B was used at concentrations of 200 μ g/mL. *Saccharomyces cerevisiae* cells were grown in synthetic defined (SD) broth without uracil (-ura) shaking aerobically or on SD-ura agar plates at 30°C.

Vector construction

Tn7:*lacZ* plasmids

Mini-Tn7 plasmids for each β -galactosidase reporter were created from a plasmid derived from pMP749 (LoVullo et al., 2009). *E. coli lacZ* was amplified from pEX-*pigR::lacZ* (Charity et al., 2009) using a 5' primer specifying a NotI site and alanine linker (5'-GCGGCCGCT-3') and a 3' primer specifying a BamHI site. The amplified *lacZ* gene was cloned into NotI/BamHI digested pMP749, resulting in pKR68 (Tn7-*lacZ*). Subsequently, two fragments were amplified from LVS genomic DNA (gDNA): (1) the *tul4* promoter with a 5' primer specifying a KpnI site

and a 3' primer overlapping the second fragment; and (2) either modified or wild-type UTRs from genes of interest, along with the first six codons of the corresponding gene, with a 3' primer specifying a NotI site and a 5' primer overlapping the first fragment. Overlap extension PCR was then conducted on the two fragments and the PCR product was cloned into KpnI/NotI digested pKR68 such that *lacZ* was in-frame with the first six codons of the gene of interest. The resulting plasmids are all indicated in **Table 1** below. Modifications to wild-type UTRs were encoded on primers for PCR amplification.

Some reporter plasmids with the high-copy pUC *ori* produced enough β -galactosidase in *E. coli* to be toxic, so cloning required one of two alternate approaches. In one approach, the origin of the pMP749 plasmid was replaced by a low-copy R6Ky origin, amplified from pKL91 (Ramsey et al., 2020) using primers that encode an NspI site. The digested product was cloned into NspI-digested pMP749, resulting in pKR88 (Mini_Tn7_R6Kg), which was propagated in DH5 α λ -pir cells. Subsequently, the *tul4* promoter and 5' UTR was amplified from LVS gDNA using a 5' primer specifying a KpnI and a 3' primer specifying a NotI site; *lacZ* was amplified from pKR68 using a 5' primer specifying a NotI site and alanine linker (5'-GCGGCCGCT-3') and a 3' primer specifying a BamHI site. The two fragments were cloned into BamHI/KpnI-digested pKR88 using a three-way ligation, resulting in pKR89 (Tn7_P*tul4*_tul4UTR_*lacZ*_R6Kg; **Table 1**).

In a second approach, *lacZ* plasmids were cloned using *Saccharomyces cerevisiae*. The 2 μ origin and *URA3* gene were isolated from pYES2 (Invitrogen) by digestion with PstI, then cloned into DraI-digested pKR68, disrupting the β -

lactamase gene. The resulting plasmid, pKR128 pYES2 Tn7-*lacZ*, was used for subsequent cloning of 5' UTRs using alanine linkers and NotI sites, as described above and detailed in **Table 1** below. pYES2-based plasmids were purified from overnight cultures of *S. cerevisiae* using the Zymoprep Yeast Plasmid Miniprep III kit.

pF-GFP plasmids

Multicopy GFP reporter plasmids were created from a previously described shuttle vector, pFNLTP6 (Maier et al., 2004). A fragment containing the promoter, 5' UTR, and first six codons of *tul4* was digested from pKR89 with KpnI/NotI. sfGFP codon-optimized for expression in *F. tularensis* LVS was purchased as a gBlock (IDT) and digested with NotI/BamHI. Fragment were cloned into KpnI/BamHI-digested pF such that GFP was in-frame with the first six codons of *tul4*, resulting in pKR145 (pF-*tul4* UTR-GFP). The plasmid pKR146 (pF-*pdpA* UTR-GFP) was constructed similarly, after amplification from pKR74 of the *tul4* promoter and *pdpA* 5' UTR and first six codons and digestion of the PCR product with Kpn/NotI. Subsequent constructs were cloned into pKR145 to replace the *tul4* 5' UTR using the endogenously encoded PacI site in the *tul4* promoter (**Table 1** for details). For genes in which a transcription start site had not been annotated at the time of plasmid design, 100 nucleotides upstream of the start codon were included as the 5' UTR (**Table 1** for details). Known transcription start sites for *tul4*, *iglA*, and *pdpA* were previously published by Ramsey et al. (2015); the transcription start site for *hfq* was experimentally determined by Meibom et al. (2009) and Chambers & Bender (2011).

Allelic exchange plasmid

The plasmid pEX18kan was modified to create the in-frame deletion construct for deletion of *hfq* as previously described (Charity et al., 2007). Flanking regions of ~1000 base pairs from both sides of the *hfq* gene were amplified by PCR. Primers amplifying the DNA adjacent to *hfq* 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, yielding pKL111 pEXΔ*hfq*.

VSV-G tagging integration vector

A single-integration vector for VSV-G tagging of *hfq* was made by modifying pKL02 (Ramsey et al., 2015). The final 200 nucleotides of the 3' end of *hfq* was amplified using a 5' primer specifying a KpnI site and a 3' primer that lacked the native stop codon and included DNA specifying a NotI site. The fragment was cloned into KpnI/NotI digested pKL02 such that the 3' end of *hfq* is in frame with the codons specifying three alanine residues followed by the VSV-G epitope, resulting in pKR158 (pEX-*hfq*-V).

Strain construction

β -galactosidase reporter strains (**Table 2**) were constructed by site- and orientation-specific single chromosome integration using the Tn7 transposon as previously described (LoVullo et al., 2009). Helper plasmid pMP720 was electroporated into either wild-type (LVS) or bS21-2 mutant ($\Delta rpsU2$) competent cells in 0.2 cm cuvettes with a 2.5 kV pulse and hygromycin-resistant cells were selected by plating on CHA-H with hygromycin. Cells with the helper plasmid were electroporated with the appropriate mini-Tn7 plasmid and selected for on CHA-H with kanamycin. Colonies were screened for plasmid integration at the *attTn7* site using PCR. Candidate strains were confirmed by amplification of genomic DNA outside of the *attTn7* site and Sanger sequencing (Rhode Island INBRE Molecular Informatics Core).

Reporter constructs encoded on pF plasmids were electroporated into LVS, LVS $\Delta rpsU2$, or LVS Δhfq cells as described above and selected for on CHA-H with kanamycin (**Table 2**).

The Hfq deletion strain was constructed by allelic exchange as previously described (Trautmann & Ramsey 2022). Briefly, at least 1 μ g of allelic exchange plasmid pEX Δhfq was electroporated into competent cells as above. Cells in which a single integration event occurred were selected for on CHA-H-kanamycin. Counter-selection for the vector was accomplished by plating on CHA-H (BD Difco) containing 10% sucrose. Sucrose-resistant, kanamycin-sensitive colonies 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, validating LVS Δhfq .

Cells with VSV-G-tagged Hfq were made as previously described (Ramsey et al., 2015). Briefly, at least 1 μ g of pKR158 pEX-*hfq*-V was electroporated into LVS and $\Delta rpsU2$ cells and transformants were selected on CHA-H-with kanamycin. Cells were confirmed to have a single integration by PCR amplification of DNA across the integration site and subsequent Sanger sequencing of the PCR product (RI-INBRE CRCF).

Table 2: Strains used in this study

Strain Number	Description	Background Strain	Plasmid Name
β-galactosidase reporter strains			
KRLVS96	LVS Tn7::Ptul4-pdpA 5'UTR-lacZ aphA	LVS	pKR74
KRLVS97	LVS ΔrpsU2 Tn7::Ptul4-pdpA 5'UTR-lacZ aphA	ΔrpsU2	pKR74
KRLVS102	LVS Tn7::Ptul4-pdpA 5'UTR-mut1-lacZ aphA	LVS	pKR84
KRLVS106	LVS ΔrpsU2 Tn7::Ptul4-pdpA 5'UTR-mut1-lacZ aphA	ΔrpsU2	pKR84
KRLVS110	LVS Tn7::Ptul4-pdpA 5'UTR-mut2-lacZ aphA	LVS	pKR85
KRLVS107	LVS ΔrpsU2 Tn7::Ptul4-pdpA 5'UTR-mut2-lacZ aphA	ΔrpsU2	pKR85
KRLVS114	LVS Tn7::Ptul4-pdpA 5'UTR-badSD-lacZ aphA	LVS	pKR98
KRLVS117	LVS ΔrpsU2 Tn7::Ptul4-pdpA 5'UTR-badSD-lacZ aphA	ΔrpsU2	pKR98
KRLVS115	LVS Tn7::Ptul4-pdpA 5'UTR-ideal_movedSD-lacZ aphA	LVS	pKR99
KRLVS118	LVS ΔrpsU2 Tn7::Ptul4-pdpA 5'UTR-ideal_movedSD-lacZ aphA	ΔrpsU2	pKR99
KRLVS112	LVS Tn7::Ptul4-tul4 5'UTR-lacZ aphA	LVS	pKR89
KRLVS111	LVS ΔrpsU2 Tn7::Ptul4-tul4 5'UTR-lacZ aphA	ΔrpsU2	pKR89
KRLVS158	LVS Tn7::Ptul4-pdpA 5'UTR-idealSD-lacZ aphA	LVS	pKR129
KRLVS160	LVS ΔrpsU2 Tn7::Ptul4-pdpA 5'UTR-idealSD-lacZ aphA	ΔrpsU2	pKR129
KRLVS159	LVS Tn7::Ptul4-pdpA 5'UTR-tul4SD-lacZ aphA	LVS	pKR130
KRLVS161	LVS ΔrpsU2 Tn7::Ptul4-pdpA 5'UTR-tul4SD-lacZ aphA	ΔrpsU2	pKR130
pF-GFP reporter strains			
KRLVS180	LVS pF-tul4UTR-GFP	LVS	pKR145
KRLVS182	LVS ΔrpsU2 pF-tul4UTR-GFP	ΔrpsU2	pKR145

Strain Number	Description	Background Strain	Plasmid Name
KRLVS234	LVS Δhfq pF- <i>tul4</i> UTR-GFP	Δhfq	pKR145
KRLVS181	LVS pF- <i>pdp</i> AUTR-GFP	LVS	pKR146
KRLVS183	LVS $\Delta rpsU2$ pF- <i>pdp</i> AUTR-GFP	$\Delta rpsU2$	pKR146
KRLVS236	LVS Δhfq pF- <i>pdpA</i> UTR-GFP	Δhfq	pKR146
KRLVS188	LVS pF- <i>mraY</i> UTR-GFP	LVS	pKR151
KRLVS189	LVS $\Delta rpsU2$ pF- <i>mraY</i> UTR-GFP	$\Delta rpsU2$	pKR151
KRLVS190	LVS pF-FTL_0215UTR-GFP	LVS	pKR152
KRLVS191	LVS $\Delta rpsU2$ pF-FTL_0215UTR-GFP	$\Delta rpsU2$	pKR152
KRLVS199	LVS pF- <i>mraY</i> UTR_mut1-GFP	LVS	pKR156
KRLVS200	LVS $\Delta rpsU2$ pF- <i>mraY</i> UTR_mut1-GFP	$\Delta rpsU2$	pKR156
KRLVS201	LVS pF- <i>mraY</i> UTR_mut2-GFP	LVS	pKR157
KRLVS202	LVS $\Delta rpsU2$ pF- <i>mraY</i> UTR_mut2-GFP	$\Delta rpsU2$	pKR157
KRLVS206	LVS pF- <i>iglA</i> UTR-GFP	LVS	pKR160
KRLVS207	LVS $\Delta rpsU2$ pF- <i>iglA</i> UTR-GFP	$\Delta rpsU2$	pKR160
KRLVS208	LVS pF-FTL_0222UTR-GFP	LVS	pKR161
KRLVS209	LVS $\Delta rpsU2$ pF-FTL_0222UTR-GFP	$\Delta rpsU2$	pKR161
KRLVS210	LVS pF-FTL_0881UTR-GFP	LVS	pKR162
KRLVS211	LVS $\Delta rpsU2$ pF-FTL_0881UTR-GFP	$\Delta rpsU2$	pKR162
KRLVS212	LVS pF-FTL_1093UTR-GFP	LVS	pKR163
KRLVS213	LVS $\Delta rpsU2$ pF-FTL_1093UTR-GFP	$\Delta rpsU2$	pKR163
KRLVS214	LVS pF- <i>mraY</i> UTR_mut5-GFP	LVS	pKR165
KRLVS215	LVS $\Delta rpsU2$ pF- <i>mraY</i> UTR_mut5-GFP	$\Delta rpsU2$	pKR165
KRLVS222	LVS pF- <i>mraY</i> UTR_idealSD-GFP	LVS	pKR169
KRLVS223	LVS $\Delta rpsU2$ pF- <i>mraY</i> UTR_idealSD-GFP	$\Delta rpsU2$	pKR169
KRLVS228	LVS pF- <i>hfq</i> UTR-GFP	LVS	pKR172

Strain Number	Description	Background Strain	Plasmid Name
KRLVS229	LVS $\Delta rpsU2$ pF- <i>hfq</i> UTR-GFP	$\Delta rpsU2$	pKR172
KRLVS243	LVS pF- <i>mra</i> YUTR_mut6-GFP	LVS	pKR175
KRLVS244	LVS $\Delta rpsU2$ pF- <i>mra</i> YUTR_mut6-GFP	$\Delta rpsU2$	pKR175
KRLVS247	LVS pF- <i>mra</i> YUTR_mut7-GFP	LVS	pKR177
KRLVS248	LVS $\Delta rpsU2$ pF- <i>mra</i> YUTR_mut7-GFP	$\Delta rpsU2$	pKR177
KRLVS252	LVS pF- <i>mra</i> YUTR_mut8-GFP	LVS	pKR179
KRLVS253	LVS $\Delta rpsU2$ pF- <i>mra</i> YUTR_mut8-GFP	$\Delta rpsU2$	pKR179
KRLVS260	LVS pF- <i>mra</i> YUTR_mut9-GFP	LVS	pKR180
KRLVS261	LVS $\Delta rpsU2$ pF- <i>mra</i> YUTR_mut9-GFP	$\Delta rpsU2$	pKR180
KRLVS262	LVS pF- <i>mra</i> YUTR_mut3-GFP	LVS	pKR182
KRLVS263	LVS $\Delta rpsU2$ pF- <i>mra</i> YUTR_mut3-GFP	$\Delta rpsU2$	pKR182
KRLVS264	LVS pF- <i>mra</i> YUTR_mut4-GFP	LVS	pKR183
KRLVS265	LVS $\Delta rpsU2$ pF- <i>mra</i> YUTR_mut4-GFP	$\Delta rpsU2$	pKR183
Deletion strains			
KMLFT97	LVS Δhfq	LVS	pKL111
VSV-G tagged strains			
KRLVS194	LVS <i>hfq</i> -VSVG	LVS	pKR158
KRLVS195	LVS $\Delta rpsU2$ <i>hfq</i> -VSVG	$\Delta rpsU2$	pKR158

β-galactosidase assays

β-galactosidase assays using *F. tularensis* LVS or *ΔrpsU2* cells containing indicated reporter constructs were conducted as previously described (Charity et al., 2009). If significant yellow color was not produced within two hours, reactions were stopped at 120 minutes. Experiments were conducted at least twice in biological triplicate.

GFP assays

F. tularensis LVS, *ΔrpsU2*, or *Δhfq* reporter constructs were grown in sMHB to mid-log phase in biological triplicate. Cells were pelleted and resuspended in PBS. A₆₀₀ and fluorescence with excitation of 495 nm and emission of 535 nm were determined using ID3 plate reader (RI-INBRE CRCF), in technical triplicate. Fluorescence readings were normalized to A₆₀₀ and fluorescence of LVS cells (lacking any GFP reporter) was subtracted from each reading to account for basal level fluorescence of the cells. Experiments were conducted at least twice in biological triplicate.

Plasmid copy number qPCR

Wild-type LVS or LVS *ΔrpsU2* cells with pF plasmids were grown to mid-log (OD₆₀₀ = 0.3-0.4). Total DNA was extracted from 1 mL culture using the MasterPure Complete DNA purification kit (Lucigen). qRT-PCR was performed using PowerUp SYBR Green Master Mix (Applied Biosystems) and a Roche LightCycler 480 (RI-INBRE CRCF) essentially as described (Charity et al., 2007) with 0.05 ng of DNA. DNA abundances were calculated for an opening reading frame (ORF3) on the plasmid and relative abundance is reported compared to a

chromosomally-encoded control gene, *tul4*. Experiments comparing wild-type and *rpsU2* mutant cells were performed three times in biological triplicate.

5' UTR secondary structure prediction

Secondary structures reported above were predicted using the MXfold2 web server (Sato et al., 2021). Cross-algorithm comparison of predictions (Figure S4) were conducted to validate findings using the ViennaRNA 2.0 package via Snapgene v6.2 (Lorenz et al., 2011), the MXFold2 server, and Ufold v1.2 webserver (Fu et al., 2022).

5' UTR motif analyses

The 5' UTRs of genes that have decreases in protein, but not transcript, abundance in cells lacking bS21-2 compared to wild-type (Trautmann & Ramsey, 2022) were analyzed. As there is insufficient annotation of transcriptional start sites in *F. tularensis* LVS, 100 nucleotides upstream of the start codon along with the first six codons were chosen for analysis.

STREME software (MEME suite) was used to analyze the 5' UTRs of the 20 genes with the largest fold decreases in protein in $\Delta rpsU2$ cells compared to LVS. These were compared to shuffled sequences to find two candidate motifs. As a control, the same parameters were used to compare the predicted 5' UTRs of 20 genes not differentially expressed in LVS and $\Delta rpsU2$.

Shine-Dalgarno predictions were made by highest similarity to the reverse complement of the anti-Shine-Dalgarno (5'-AGGAGG-3') within 20 nucleotides of the start codon.

Immunoblotting

Immunoblotting was completed as previously described (Trautmann & Ramsey, 2022). Briefly, cell lysates were separated by SDS-PAGE, transferred to PVDF, and analyzed for total protein with the Invitrogen No-Stain Protein labeling reagent for normalization. Membranes were probed with indicated monoclonal antibodies (BEI Resources, diluted 1:250 for PdpB, 1:1000 for IgIB, and 1:1000 for IgIA) or the VSV-G epitope tag (Sigma, diluted 1:2222) in blocking buffer. Proteins were detected using IRDye 800 CW donkey anti-mouse IgG or donkey anti-rabbit IgG secondary antibodies (Li-Cor, diluted 1:10,000). Protein abundance was calculated as fluorescence of protein bands relative to total protein in each lane. Experiments were performed at least twice in biological triplicate.

RNA purification and qRT-PCR

Total RNA was purified according to the RNAsnap™ protocol (Stead et al., 2012). *F. tularensis* LVS was grown in biological triplicate to mid-log phase. Pelleted cells (10 mL) were resuspended in 100 µl of fresh RES (95% formamide, 18 mM EDTA, 0.025% SDS, 1% BME), then incubated at 95°C for 7 minutes. Cell debris were pelleted by centrifugation and the supernatant was preserved. Total nucleic acid was recovered with 0.3M sodium acetate (pH 5.2) and 3x volumes 100% ethanol. Samples were stored at -80°C for 1 hour, then nucleic acid was pelleted by centrifugation at 4°C for 30 minutes. The pellet was washed with 75% ethanol and resuspended in water. Purified nucleic acids were treated with RQ1 DNase (Promega) for 1 hour at 37°C and RNA was purified again with sodium acetate/ethanol precipitation.

cDNA was synthesized using Superscript III reverse transcriptase (Life Technologies) as previously described (Charity et al., 2007). qRT-PCR was performed with the PowerUP SYBR Green Master Mix (Applied Biosystems) and the Roche Lightcycler 480 (RI-INBRE CRCF). Transcript abundances of *pdpA*, *pdpB*, *iglA*, *pigR*, *rpoA1*, and *bfr* were normalized to a control gene, *tul4*. Experiments were conducted twice in biological triplicate.

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