

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

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Abstract

Although ribosomes are generally examined in aggregate, ribosomes can be heterogenous in composition. Evidence is accumulating that changes in ribosome composition may result in altered function, such that ribosome heterogeneity may provide a mechanism to regulate protein synthesis. Ribosome heterogeneity in the human pathogen *Francisella tularensis* results from incorporation of one of three homologs of bS21, a small ribosomal subunit 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. Here, we examine the potential for bS21-2 to function in a leader sequence-dependent manner and to function indirectly, via Hfq. We found that the 5' untranslated region (UTR) of some bS21-2-responsive genes, including key virulence genes, is sufficient to alter translation in cells lacking bS21-2. We further identify features of a 5' UTR that allow responsiveness to bS21-2. These include an imperfect Shine-Dalgarno sequence and a particular 6-nucleotide sequence. Our results are consistent with a model in which a bS21 homolog increases efficiency of translation initiation through interactions with specific leader sequences. With respect to bS21-2 indirectly regulating translation via the RNA-binding protein Hfq, we found that Hfq controls transcript abundance rather than protein synthesis, impacting virulence gene expression via a distinct mechanism. Together, we determined that ribosome composition in *F. tularensis* regulates translation in a leader sequence-dependent manner, a regulatory mechanism which may be used in other bacteria.

Importance

23 Ribosome heterogeneity is common in bacteria and there is mounting evidence that
24 ribosome composition plays a regulatory role in protein synthesis. However, mechanisms of
25 ribosome-driven gene regulation are not well understood. In the human pathogen *Francisella*
26 *tularensis*, which encodes multiple homologs for the ribosomal protein bS21, loss of one
27 homolog impacts protein synthesis and virulence. Here, we explore the mechanism behind
28 bS21-mediated changes in protein synthesis, finding that they can be linked to altered
29 translation initiation and are dependent on specific sequences in the leaders of transcripts. Our
30 data support a model in which ribosome composition regulates gene expression through
31 translation, a strategy that may be conserved in diverse organisms with various sources of
32 ribosome heterogeneity.

33 Introduction

34 Ribosomes, the molecular machines that synthesize proteins, can be heterogeneous in
35 composition (1). As bacterial ribosomes are composed of 3 ribosomal RNA molecules (rRNAs)
36 and ~ 50 ribosomal proteins (r-proteins), heterogeneity can arise from differences in rRNA
37 sequence among *rrn* operons, rRNA or r-protein modification, or r-protein content (2). The
38 consequences of ribosome heterogeneity are incompletely understood, and much debate
39 surrounds the hypothesis that distinct classes of ribosomes can have specialized functions by
40 preferentially translating subsets of mRNA (3).

41 In *Escherichia coli*, the 30S subunit r-protein bS21 is not required for translation *in vitro*
42 but is essential for viability (4, 5). It is one of the last proteins to be incorporated during 30S
43 subunit assembly (6). While the precise function of bS21 in translation is not clear, early
44 studies demonstrated its involvement in translation initiation (7, 8, 9). Association of bS21 with
45 the 30S subunit is reversible, as it is easily exchanged among ribosomes (10) suggesting that
46 the presence or absence of bS21 can act as a source of ribosomal heterogeneity.

47 More recently, multiple studies have suggested that bS21 might play a regulatory role in
48 gene expression (11, 12; 13; 14). High-resolution ribosome structures have shown bS21 to be
49 located in the 30S subunit platform, near the mRNA exit channel, and in position to interact
50 with the anti-Shine-Dalgarno (ASD) sequence of 16S rRNA in the 30S initiation complex (15)
51 and with the Shine-Dalgarno/anti-Shine-Dalgarno (SD-ASD) helix in the 70S ribosome (16),
52 suggesting possible mechanisms for regulation.

53 Recent work in the Bacteroidia species *Flavobacterium johnsoniae* clearly demonstrates
54 that bS21 controls gene expression. In this organism, incorporation of the single bS21
55 homolog into the ribosome contributes to sequestration of the ASD (12). The mRNA encoding

56 bS21, *rpsU*, is essentially the only *F. johnsoniae* mRNA with a strong SD sequence. Depletion
57 of bS21 or removal of the region of bS21 necessary for ASD sequestration results in increased
58 translation from the *rpsU* mRNA and mRNAs engineered to have a strong SD (17). These
59 studies unambiguously demonstrate that ribosomes lacking bS21 have altered specificity for
60 particular mRNAs in translation initiation, providing evidence that bS21 functions as a bona
61 fide regulator of gene expression (17).

62 *Francisella tularensis*, a human pathogen that requires a type VI secretion system
63 (T6SS) to cause disease, encodes three distinct homologs of bS21. We have shown that all
64 three homologs can be incorporated into ribosomes (14). Accordingly, in *F. tularensis*,
65 ribosome heterogeneity may be due to the identity of the bS21 homolog incorporated or, as in
66 *F. johnsoniae*, the presence or absence of bS21 in the ribosome. Importantly, loss of one of
67 the homologs, bS21-2, leads to reductions in most T6SS proteins that cannot be explained by
68 changes in transcript abundance or protein stability. Loss of bS21-2 also results in defective
69 intramacrophage replication in cells that can be complemented by restoration of bS21-2, but
70 not by either of the other two homologs. These results indicate that bS21-2 specifically governs
71 translation of virulence genes, including those that encode the T6SS (14).

72 While our observations support a model in which bS21 proteins in *F. tularensis* regulate
73 gene expression at the level of translation, they are not likely to exert their effects in the same
74 manner as the bS21 homologs in Bacteroidia. In *F. johnsoniae*, the C-terminal region of the
75 single bS21 homolog is required to interact with and sequester the ASD; while this region is
76 conserved amongst the Bacteroidia it is not conserved among other bacterial lineages,
77 including in Gammaproteobacteria like *F. tularensis* (12). Thus, in the current study we aim to
78 understand the mechanisms by which bS21-2 affects translation in *F. tularensis*.

79 Through reporter assays using translational fusions, we find that the 5' untranslated
80 region (5' UTR) of some transcripts is sufficient to cause differences in protein production if
81 bS21-2 is lost, indicating that these 5' UTRs are responsive to bS21-2. By mutagenizing the
82 bS21-2-responsive 5' UTRs, we also found that transcripts with ideal SD sequences do not
83 require bS21-2 for efficient translation. In an attempt to identify which component of the 5' UTR
84 is driving the responsiveness to bS21-2, we determined that two motifs enriched in bS21-2-
85 controlled genes are not needed to cause changes in protein abundance and that the
86 secondary structure of leader sequences do not play a clear role in responsiveness to bS21-2.
87 Yet we have identified a short nucleotide sequence in the 5' UTR of *mraY* that is critical for
88 control by bS21-2. Finally, using qPCR and immunoblot analyses, we show that bS21-2 and
89 the RNA-binding protein Hfq both govern T6SS protein abundance but do not act in a
90 coordinated manner and function via distinct pathways. Our findings suggest that the r-protein
91 bS21-2 governs protein abundance in *F. tularensis* by influencing translation from mRNA
92 species with specific leader sequences.

Results

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, in the ribosome, is located adjacent to the 5' untranslated region (UTR) of mRNAs during translation initiation (9, 15). Loss of bS21-2 in *F. tularensis* leads to changes in abundance for a subset of the proteome (14). 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. To accomplish this, we developed a series of reporter constructs consisting 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 1A**). Reporter constructs were expressed from the *tul4* promoter, which is unaffected by the presence or absence of bS21-2 (14). This design allows for comparable transcription of reporter genes in both genotypes to compare relative translation initiation. The reporter constructs were introduced into either wild-type *F. tularensis* (WT) or *F. tularensis* lacking 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 genes with significant changes in protein abundance in cells lacking bS21-2 (14). Consistent

116 with the observed changes in protein abundance being due to changes in translation initiation,
117 we found that the 5' UTRs of *pdpA*, *iglA*, *mraY*, FTL_0222, or FTL_1093 genes fused to *gfp*
118 led to significantly less fluorescence in cells lacking bS21-2 compared to wild-type (**Fig 1B**). In
119 contrast, the 5' UTR of *tul4*, a gene not differentially expressed in cells lacking bS21-2, did not
120 lead to a significant decrease in fluorescence in cells lacking bS21-2 (**Fig 1B**). These data
121 reveal that the 5' UTR of a gene is sufficient for bS21-2 to affect translation and is consistent
122 with the idea that bS21 may be regulating translation initiation. We will refer to 5' UTRs that
123 result in altered protein abundance in the presence of bS21-2 as "bS21-2-responsive." We also
124 found that the 5' UTRs of some genes governed by bS21-2 in our proteomics analysis did not
125 lead to reporter activity differences in cells lacking bS21-2, including FTL_0881 and FTL_0215
126 (**Fig S2**). We do not have experimentally determined transcription start sites for these genes,
127 so it is possible the lack of regulation is due to inaccurate 5' UTR predictions. Other
128 possibilities, including indirect regulation of these particular genes, are described in the
129 discussion. We confirmed that alterations in protein production in cells lacking bS21-2 could be
130 complemented by ectopic expression of bS21-2 from a plasmid, indicating that the changes in
131 translation are not due to polar effects of the *rpsU2* deletion (**Fig 1C**).

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

133 Based on structures of the *E. coli* ribosome during translation initiation, the bS21
134 residue R17 is close enough to directly contact the 16S rRNA nucleotide C1539, which is part
135 of the anti-Shine-Dalgarno sequence (15; **Fig 2A**). R17 is conserved in all three *F. tularensis*
136 bS21 homologs and the rRNA-encoded ASD is identical in *F. tularensis* and *E. coli*. Thus, we
137 hypothesized that bS21 homologs in *F. tularensis* may also contact the ASD and influence SD
138 binding during translation initiation.

139 First, we assessed how SD strength correlates with responsiveness to bS21-2. We
140 compared predicted SDs for genes whose proteins are positively affected (n=74), negatively
141 affected (n=84), or unaffected (n=82) by bS21-2 (14). We found that the genes positively
142 affected by bS21-2 generally have weaker SD sequences, with only 39% having strong SD
143 sequences (defined by 4 or more nucleotides [nt] complementary to the ASD), compared to
144 54% or 69% strong SDs in negatively affected or unaffected genes, respectively (**Fig 2B**).
145 These data are consistent with a model in which bS21-2 increases translation initiation
146 predominately in the absence of strong SD-ASD interactions.

147 We specifically examined the influence of the SD sequences on the bS21-2-responsive
148 5' UTR of the FPI gene *pdpA*. In particular, we developed β -galactosidase translational
149 reporters with altered SD sequences in the *pdpA* 5' UTR (**Fig 2C**). 5' UTRs with mutations that
150 retained imperfect base-pairing between the ASD and SD (badSD, *tu/4SD*) were still bS21-2-
151 responsive. However, introducing an ideal SD, in two different positions (idealSD,
152 ideal_movedSD), led to similar reporter gene expression in cells with and without bS21-2,
153 indicating that these 5' UTRs are no longer bS21-2-responsive (**Fig 2C**).

154 We replicated the impact of a perfect SD on the bS21-2-responsive 5' UTR of another
155 gene, *mraY*. Modification of the imperfect *mraY* SD to an ideal SD resulted in no significant
156 difference in GFP production in cells with or without bS21-2 (**Fig 3A**). It is worth noting that in
157 each of these cases, the addition of a perfect SD in the correct location (separated from the
158 start codon by 4 to 9 nt) leads to increased total reporter production (**Fig S3, S4**). These data
159 suggest that genes with perfect SD sequences do not require bS21-2 for efficient translation; in
160 other words, an ideal SD may lead to such efficient translation that any response to bS21-2

161 becomes negligible. However, given that many *F. tularensis* genes have weak or non-perfect
162 SDs and are not affected by bS21-2, there is an unidentified component of the 5' UTR that
163 results in responsiveness to bS21-2.

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

166 We reasoned that 5' UTRs may be responsive to bS21-2 because they harbor a
167 common sequence-specific element that mediates an altered interaction with bS21-2 or bS21-
168 2-containing ribosomes. To identify such an element, we compiled 5' UTR sequences
169 including 100 nts upstream of the start codon and the first six codons of the gene for all
170 proteins that were significantly less abundant in cells lacking bS21-2 compared to wild-type
171 (n=74; 100 nt was arbitrarily chosen because most *F. tularensis* transcription start sites have
172 not been identified). As a control, we also compiled 5' UTR sequences from 82 genes that
173 were not impacted by bS21-2 presence. Using the motif-finding algorithm STREME, which
174 identifies ungapped motifs enriched in large data sets (18), we identified sequence motifs
175 enriched in the 5' UTRs of the 20 genes most positively governed by bS21-2 (14). The two
176 motifs we identified were enriched compared to shuffled sequences and were not found to be
177 enriched in the control sequences. These motifs, which we refer to as Motif 1 and Motif 2, are
178 AU-rich (Motif 1 consensus AAAAUAAA and Motif 2 consensus UUAUUUA) and are found in
179 19 and 18 of the 20 sequences assessed, respectively (**Fig S5**).

180 The *mraY* 5' UTR contains sequences corresponding to both Motifs 1 and 2, so we
181 generated targeted mutations to these sequences to assess their impact on responsiveness to
182 bS21-2. Mutations 1 and 2 modified Motif 1 from AAAAUAAC to CCCC GCCG, which altered

183 the AU-content of the entire motif, and AAAUAUACA, which altered the three most conserved
184 nt in the motif. When assessed using the GFP reporter assay, neither of these modifications
185 altered the responsiveness of the 5' UTR to bS21-2 (**Fig 3B, 3F**). To assess the contribution of
186 Motif 2, we created mutation 5, a truncation of the 5' end of the *mraY* 5' UTR that removed 25
187 nt including Motif 2 (**Fig 3F**). This mutant 5' UTR also remained responsive to bS21-2 (**Fig**
188 **3C**). We additionally created mutation 10, a truncation of the 5' end of the *mraY* 5' UTR which
189 removes both Motif 1 and 2 (**Fig 3F**). Notably, this mutation led to loss of responsiveness to
190 bS21-2 (**Fig 3B**). Together, these data indicate that while neither of the two STREME-
191 predicted AU-rich motifs are necessary for the positive impact of bS21-2 on translation of the
192 *mraY* 5' UTR, some sequence between Motifs 1 and 2 is key for bS21-2-responsiveness.

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

194 While testing the importance of motifs enriched in bS21-2-responsive 5' UTRs to
195 responsiveness of the *mraY* 5' UTR, our analysis revealed that the region key for bS21-2-
196 responsiveness is between nucleotides 52 – 75 upstream from the initiation codon. To further
197 clarify the sequence necessary for bS21-2-responsiveness in the *mraY* 5' UTR, we made a
198 series of truncations and modifications from the 5' end of the leader sequence. Modifying the
199 AU-rich region located 64 – 70 nt from the initiation codon (*mraY* mut8) did not impact bS21-2-
200 responsiveness (**Fig 3C, F**). But truncating the 5' UTR to 57 nt (*mraY* mut6) led to loss of
201 bS21-2-responsiveness, implicating the nucleotides 58-63 upstream of the initiation codon,
202 GACUCU, in responsiveness to bS21-2 (**Fig 3C, F**). We further assessed the importance of
203 the GACUCU sequence using *mraY* mut7 (truncating the 5' UTR to 60 nt and changing the
204 first three nucleotides to AGA) and *mraY* mut9 (truncating the 5' UTR to 63 nt and mutating
205 nucleotides 58-63 to AGUGAG) and found neither was responsive to bS21-2 (**Fig 3C, F**).

206 These data allow us to conclude that the nucleotides 58-63 upstream of the *mraY* initiation
207 codon, GACUCU, are critical for bS21-2-responsive translation of the *mraY* 5' UTR. This is
208 consistent with a model in which bS21-2-containing ribosomes interact directly or indirectly
209 with a specific element of the leader sequence to facilitate efficient translation initiation on
210 some transcripts.

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

213 The secondary structure of mRNA molecules is an important determinate of translation
214 initiation efficiency (19, 20). We considered the possibility that the key region necessary for
215 bS21-2-responsiveness, the GACUCU sequence found 58-63 nt upstream of the *mraY*
216 initiation codon, is important because it is part of a secondary structure recognized by bS21-2-
217 containing ribosomes. In fact, in the predicted secondary structure of the *mraY* 5' UTR, the key
218 GACUCU sequence, base-pairs with the sequence 18 – 23 nt upstream of the initiation codon
219 and 3 nt upstream from the predicted SD sequence (**Fig 3D**). This base-pairing forms a stem-
220 loop structure dependent on the key GACUCU sequence that could be found in close proximity
221 to bS21 during translation initiation. To test the relevance of this potential structure, we
222 mutated the sequence predicted to base-pair with the key region for bS21-2-responsiveness,
223 at -23 – -18, thereby disrupting the structure (*mraY* mut3) (**Fig 3D, F**). We also made
224 complementary mutations to restore the structure (*mraY* mut4) (**Fig 3D, F**) and assessed these
225 5' UTRs in a GFP reporter assay (**Fig 3E**). We found that the disruption to the predicted
226 secondary structure (*mraY* mut3) did not affect bS21-2-responsiveness. However, the
227 complementary mutation that altered the previously identified GACUCU sequence but restored
228 the stem-loop structure, was no longer responsive to bS21-2 (**Fig 3E**). These results reveal

229 that the GACUCU sequence, rather than the structure it participates in forming, is key for
230 conferring responsiveness of the *mraY* 5' UTR to bS21-2.

231 It is worth noting that loss of responsiveness to bS21-2 is not simply due to more or less
232 protein synthesis. While many of the modifications that result in non-responsive leader
233 sequences reduced overall reporter protein production (*mraY* mut4, 6, 7, 9), the *mraY* mut10
234 leader is similarly non-responsive but leads to increased reporter protein (**Fig S4**).

235 In contrast to the *mraY* 5' UTR, the *pdpA* 5' UTR does not contain the sequence
236 GACUCU, nor is it long enough to contain sequence at a conserved distance from the initiation
237 codon (GACUCU is 58 – 63 nt upstream of the initiation codon; the *pdpA* 5' UTR is 24 nt long).
238 We considered the possibility that, in contrast to the *mraY* 5' UTR, a structural element of the
239 *pdpA* 5' UTR might confer responsiveness to bS21-2. We predicted the secondary structure of
240 the *pdpA* 5' UTR using MXfold2, made targeted mutations to disrupt the predicted stem-loop
241 structure (*pdpA* mut1), and generated β -galactosidase reporters at the Tn7 site in cells with
242 (WT) and without ($\Delta rpsU2$) bS21-2 (**Fig S6**). We also made complementary mutations to
243 restore the original predicted secondary structure without maintaining the original sequence
244 (*pdpA* mut2). In designing each mutation, we ensured that there was no significant disruption
245 to the Shine-Dalgarno or start codon. Neither of these variants that altered the *pdpA* 5' UTR
246 structure affected responsiveness to bS21-2, indicating that the secondary structure of this 5'
247 UTR does not play a role in translation modulation by bS21-2 (**Fig S6**). Thus, in our studies of
248 two different bS21-2-responsive 5' UTRs, we did not find a specific predicted secondary
249 structure that is necessary for bS21-2-responsive translation.

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

251 While we identified a key sequence for bS21-2-responsiveness in the *mraY* 5' UTR,
252 neither this sequence nor a similar sequence is found in other 5' UTRs positively impacted by
253 bS21-2 (**Table S1**), suggesting that other responsive 5' UTRs contain distinct sequences that
254 similarly confer responsiveness or that there are multiple mechanisms by which bS21-2 exerts
255 its effects. In considering the control of the T6SS proteins, we hypothesized that bS21-2 may
256 be exerting its effects indirectly, altering the translation of a regulator that directly controls
257 production of the T6SS proteins. One such candidate is the RNA-binding protein Hfq, which is
258 known to control gene expression post-transcriptionally in many organisms and has been
259 shown to impact the expression of T6SS proteins in *F. tularensis* (21, 22). Our proteomics
260 analysis identified increased Hfq in cells lacking bS21-2 compared to wild-type (5.9-fold),
261 although substantial variation in biological replicates precluded the differences from reaching
262 statistical significance (adj p=0.066; 14). Additionally, many of the bS21-2-responsive 5' UTRs
263 are AU-rich, raising the possibility that they may contain ARN motifs, known targets of Hfq. In
264 fact, one of the motifs we found enriched in bS21-2-responsive 5' UTRs , Motif 1, could
265 represent an ARN motif (**Fig S5**). This led us to hypothesize that Hfq may play a role in bS21-
266 2-mediated regulation of T6SS proteins.

267 To test further whether cells lacking bS21-2 have increased Hfq, we added DNA
268 specifying a C-terminal vesicular stomatitis virus glycoprotein (VSV-G) epitope tag to *hfq* at its
269 native locus on the chromosome, in cells with (WT) and without ($\Delta rpsU2$) bS21-2 and
270 determined relative protein abundance by quantitative immunoblotting. We found a moderate
271 increase in Hfq (about 30%, **Fig 4B**) in cells lacking bS21-2 compared to wild-type; this is

272 consistent with our prior proteomics findings, including that it is not a difference that would
273 have reached our significance threshold.

274 Given the detectable increase in Hfq abundance in cells lacking bS21-2, we sought to
275 determine if this is due to increased translation of the *hfq* mRNA. Using our reporter assay, we
276 assessed the relative translation of mRNAs containing the *hfq* 5' UTR. We found that there is
277 approximately 2-fold more GFP produced from reporter fusions with the 5' UTR of *hfq* in cells
278 lacking bS21-2, indicating this UTR leads to more efficient translation by ribosomes without
279 bS21-2 (**Fig 4C**). This suggests that the observed increase in Hfq in cells lacking bS21-2 is
280 due to increased translation initiation.

281 If the moderate increase in Hfq leads to the observed reduction in T6SS proteins when
282 bS21-2 is absent, Hfq must be acting as a repressor of the T6SS. However, inconsistent
283 results have been reported with respect to the role of Hfq in regulating the T6SS genes in *F.*
284 *tularensis*. A transcriptomic analysis of cells lacking Hfq found that one of the two Francisella
285 Pathogenicity Island (FPI) operons encoding the T6SS, the *pdpA* operon, was upregulated in
286 *hfq* mutant cells (diagram of FPI operons in Fig 4A; 21). In another report, a proteomic analysis
287 of *hfq* mutant cells found that proteins encoded by the other FPI operon, the *iglA* operon, are
288 less abundant but observed no change in the abundance of proteins encoded by the *pdpA*
289 operon (22). To clarify the role of Hfq in the regulation of T6SS protein abundance, we
290 determined the abundance of several T6SS proteins encoded on both operons in cells with
291 and without Hfq by quantitative immunoblotting (**Fig 4A, D**) (14). PdpB, encoded in the *pdpA*
292 operon, was more abundant in cells without Hfq compared to wild-type (>2-fold; $p < 0.01$), while
293 IglB and IglA, encoded on the *iglA* operon, were not impacted by the loss of Hfq (**Fig 4D**). This

294 is in contrast to cells lacking bS21-2, which contained reduced amounts of all three proteins
295 (**Fig 4D**; 14). These data suggest that Hfq regulates expression of proteins encoded by the
296 *pdpA* operon, but not the *iglA* operon, consistent with the observations of Meibom et al. (21).

297 Hfq can exert its effects through a variety of mechanisms, some of which result in
298 changes in translation initiation. To determine if the presence of *F. tularensis* Hfq, like bS21-2,
299 impacts the translation of T6SS proteins, we analyzed the ability of cells lacking Hfq to
300 translate mRNAs containing either the 5' UTRs of the T6SS protein gene *pdpA* or the control
301 gene *tul4* fused to *gfp*. We found that translation of mRNAs with the *pdpA* or *tul4* 5' UTRs are
302 not altered when Hfq is absent compared to wild-type, while translation of the mRNA with the
303 *pdpA* 5' UTR decreases if bS21-2 is absent (**Fig 4E**). These findings suggest that while bS21-
304 2-associated changes in T6SS proteins can be attributed to changes in translation initiation,
305 Hfq-associated changes in T6SS proteins cannot.

306 Since it has been proposed that Hfq represses *pdpA* operon transcript abundance (21)
307 and Hfq does not impact translation of *pdpA* in a 5' UTR-dependent manner, we hypothesized
308 that cells lacking Hfq might have increased *PdpB* due to increased *pdpB* (and *pdpA* operon)
309 transcript abundance. To test this, we compared the abundance of mRNAs isolated from wild-
310 type cells to those isolated from cells lacking bS21-2 ($\Delta rpsU2$) or Hfq (Δhfq) by qPCR, and
311 found that *pdpA* and *pdpB* transcripts have large, statistically significant increases when Hfq is
312 not present (9.9-fold and 24-fold, respectively) but the relative impact on *iglA* transcript
313 abundance is minor (2.5-fold increased) (**Fig 4F**; **Table S2**); this is in contrast to the minor
314 changes in *pdpA*, *pdpB*, and *iglA* transcripts in cells without bS21-2 (~2-fold or less).

315 Our findings indicate that Hfq exerts its effects on a subset of T6SS proteins by
316 negatively regulating their transcript abundance (**Fig 4F**). Conversely, bS21-2 influences the
317 abundance of essentially all the T6SS proteins and does so by influencing their protein
318 synthesis rather than their transcript abundance (**Fig 4E**; 14). Given that Hfq and bS21-2
319 influence distinct groups of proteins at different points in gene expression, our work does not
320 support a model in which Hfq mediates the effects of bS21-2 on the T6SS. Instead, our results
321 suggest two distinct pathways of regulation for the genes encoding the T6SS: one in which
322 bS21-2 improves efficiency of translation initiation from both operons, and one in which Hfq
323 represses transcript abundance of only the *pdpA* operon.

324

325 **Discussion**

326 In this work, we addressed two hypotheses regarding how bS21-2 exerts its effects on
327 protein synthesis. The first is that ribosomes containing bS21-2 may influence translation
328 initiation of specific transcripts in a leader sequence-dependent manner. Using reporter
329 assays, we determined that specific 5' UTR sequences are sufficient to lead to altered
330 translation in cells with or without bS21-2. In a comprehensive assessment of 5' UTR
331 elements, we found that bS21-2-responsive 5' UTRs have imperfect SD sequences and, in a
332 specific responsive 5' UTR, the presence of a particular six nucleotide sequence. Our second
333 hypothesis was that the effects of bS21-2 on the T6SS proteins may be mediated by Hfq, a
334 known regulator of T6SS proteins. However, since there have been conflicting reports
335 regarding the impacts of Hfq on the T6SS, we also examined the effects of Hfq on T6SS
336 protein and transcript abundance. Our work clearly demonstrates that Hfq is a negative
337 regulator of genes in one of the two FPI operons encoding T6SS proteins and that this

338 regulation influences transcript abundance rather than translation, consistent with and building
339 upon a prior study (21). In contrast, the positive effects of bS21-2 on essentially all T6SS
340 proteins can be attributed to differences in protein synthesis. Thus, we conclude that Hfq and
341 bS21-2 function in independent pathways to regulate the T6SS proteins. Together, these
342 results suggest that bS21-2 impacts protein synthesis by altering translation initiation on
343 mRNAs with specific leader sequences.

344 This work demonstrates that bS21-2 exerts its effects on protein synthesis in a leader
345 sequence-dependent manner and is validated in a subset of bS21-2-responsive 5' UTRs (**Fig**
346 **1B**). While loss of bS21-2 results in altered abundance for about 160 proteins (14), we expect
347 that changes in protein abundance for at least some of these may not be due to leader
348 sequence-dependent effects, but rather due to downstream or secondary effects. For example,
349 bS21-2 may directly impact synthesis of proteins that influence the abundance of other
350 proteins. Several proteases and peptidases were found to be differentially abundant in cells
351 lacking bS21-2 (14). Thus, proteins like those encoded by FTL_0881 and FTL_0215, which do
352 not have bS21-2-responsive leader sequences (**Fig S2**), may have altered abundance in
353 bS21-2 mutant cells due to changes in the abundance of proteases or protein processing
354 enzymes.

355 In our search for an element necessary for responsiveness of leader sequences to
356 bS21-2, we found that ideal SD sequences prevent responsiveness (**Fig 2C, 3A**). This
357 suggests that perfect SD-ASD complementarity leads to such efficient translation initiation that
358 any contribution of bS21-2 to translation are minor and effectively masked. It is perhaps
359 unsurprising that other regulators of translation, such as H-NS in *E. coli*, similarly function to

360 regulate translation of mRNAs with imperfect SDs (23). We suggest this effect is due to the
361 SD-ASD complementarity leading to increased stabilization of the 30S initiation complex,
362 obscuring any positive effects of bS21-2, rather than due to overall changes in protein
363 production. In particular, simply increasing translation is not sufficient to lead to loss of
364 responsiveness to bS21-2, as very similar amounts of protein are produced from reporters with
365 the *pdpA* tul4SD and *pdpA* idealSD leaders, but only *pdpA* idealSD is not responsive to bS21-
366 2 (**Fig 2C, S3**). And similarly, reducing protein synthesis is not sufficient to prevent
367 responsiveness to bS21-2. Moving the ideal SD sequence to a non-ideal location in
368 translational fusions (*pdpA* ideal_movedSD) reduces the amount of reporter protein but still
369 prevents responsiveness to bS21-2 (**Fig 2C, S3**). In this case, we suggest that while the ideal
370 SD prevents bS21-2-responsiveness, the reduced reporter abundance may be because the
371 extended spacing between the SD and start codon influences the transition from initiation to
372 elongation, affecting the rate of ribosome translocation immediately following the start codon
373 (24).

374 While a simple common element across all bS21-2-responsive leader sequences
375 remains elusive, we were able to hone in on the 6 nt sequence in the *mraY* 5' UTR that leads
376 to bS21-2-responsiveness, GACUCU (**Fig 3**). We speculate that the 6 nt GACUCU sequence
377 may be a direct binding site for bS21-2, and that a direct interaction between this site and
378 bS21-2 in the ribosome increases translation initiation of the *mraY* mRNA. However, this
379 specific sequence is not found in other leader sequences positively impacted by bS21-2
380 (including the short, 24 nt *pdpA* 5' UTR; **Table S1**). Further work will be necessary to
381 determine if this sequence is sufficient for bS21-2-responsiveness, to identify the

382 commonalities among bS21-2-responsive 5' UTRs, and to determine how bS21-2 influences
383 translation initiation on specific leader sequences.

384 While the key 6 nt sequence identified in the *mraY* 5' UTR is not universally required for
385 responsiveness to bS21-2, it does reveal that specific leader sequences lead to increased
386 translation by ribosomes with bS21-2. This is quite different from bS21 homologs in
387 Bacteroidia, which also influence translation in a sequence dependent manner, but exert their
388 effects by altering accessibility to the anti-Shine-Dalgarno sequence (12, 17). This raises the
389 possibility that other bS21 homologs, in *F. tularensis* and other organisms, may influence
390 translation in a leader-sequence dependent manner.

391 In *F. tularensis*, loss of the RNA chaperone Hfq results in defective intramacrophage
392 replication, which is essential for virulence. Yet how Hfq promotes *F. tularensis*
393 intramacrophage replication remains poorly-understood. Few small RNAs have been identified
394 in *F. tularensis* and none have been identified that are Hfq-dependent (25, 26). Our results
395 demonstrate that Hfq represses *pdpA* operon transcript abundance but does not influence
396 T6SS protein synthesis. The molecular mechanism by which Hfq exerts its effects on this
397 operon, and if it involves a small RNA, remain unclear. Regardless, the change in production
398 of T6SS components in cells lacking Hfq is consistent with the observed intramacrophage
399 growth defect, as *F. tularensis* cells overproducing the T6SS are defective for intramacrophage
400 survival (27).

401 **Materials and Methods**

402 ***Bacterial strains and growth conditions***

403 Unless otherwise noted, bacterial strains were grown as indicated. *Francisella tularensis*
404 subsp. *holarctica* Live Vaccine Strain (LVS) cells were grown in Mueller-Hinton broth (BD
405 Difco) supplemented with 0.025% iron pyrophosphate, 0.1% glucose, and 2% Isovitalex
406 (sMHB), shaking aerobically or on cystine heart agar (BD Difco or prepared in house) plates
407 with 1% hemoglobin (CHA-H) at 37°C. *Escherichia coli* XL1-Blue, DH5α (New England
408 Biolabs), and DH5α λ-pir cells were grown in lysogeny broth (LB) shaking aerobically or on LB
409 agar plates at 37°C. Kanamycin and nourseothricin were used at concentrations of 5 µg/mL (*F.*
410 *tularensis*) or 50 µg/mL (*E. coli*); hygromycin B was used at concentrations of 200 µg/mL.
411 *Saccharomyces cerevisiae* cells were grown in synthetic defined (SD) broth without uracil (-
412 ura) shaking aerobically or on SD-ura agar plates at 30°C.

413 ***Vector construction***

414 Tn7::lacZ plasmids

415 Mini-Tn7 plasmids for each β-galactosidase reporter were created from a plasmid
416 derived from pMP749 (28). *E. coli lacZ* was amplified from pEX-pigR::lacZ (29) using a 5′
417 primer specifying a NotI site and alanine linker (5′-GCGGCCGCT-3′) and a 3′ primer
418 specifying a BamHI site. The amplified *lacZ* gene was cloned into NotI/BamHI digested
419 pMP749, resulting in pKR68 (Tn7-lacZ). Subsequently, two fragments were amplified from LVS
420 genomic DNA (gDNA): (1) the *tul4* promoter with a 5′ primer specifying a KpnI site and a 3′
421 primer overlapping the second fragment; and (2) either modified or wild-type UTRs from genes
422 of interest, along with the first six codons of the corresponding gene, with a 3′ primer

423 specifying a NotI site and a 5' primer overlapping the first fragment. Overlap extension PCR
424 was then conducted on the two fragments and the PCR product was cloned into KpnI/NotI-
425 digested pKR68 such that *lacZ* was in-frame with the first six codons of the gene of interest.
426 The resulting plasmids are all indicated in **Table S3**. Modifications to wild-type UTRs were
427 encoded on primers for PCR amplification.

428 Some reporter plasmids with the high-copy pUC *ori* produced enough β -galactosidase
429 in *E. coli* to be toxic, so cloning required one of two alternate approaches. In one approach, the
430 origin of the pMP749 plasmid was replaced by a low-copy R6K γ origin, amplified from pKL91
431 (30) using primers that encode an NspI site. The digested product was cloned into NspI-
432 digested pMP749, resulting in pKR88 (Mini_Tn7_R6Kg), which was propagated in DH5 α λ -pir
433 cells. Subsequently, the *tul4* promoter and 5' UTR was amplified from LVS gDNA using a 5'
434 primer specifying a KpnI and a 3' primer specifying a NotI site; *lacZ* was amplified from pKR68
435 using a 5' primer specifying a NotI site and alanine linker (5'-GCGGCCGCT-3') and a 3'
436 primer specifying a BamHI site. The two fragments were cloned into BamHI/KpnI-digested
437 pKR88 using a three-way ligation, resulting in pKR89 (Tn7_P*tul4*_tul4UTR_ *lacZ*_R6Kg; **Table**
438 **S3**).

439 In a second approach, *lacZ* plasmids were cloned using *Saccharomyces cerevisiae*.
440 The 2 μ origin and *URA3* gene were isolated from pYES2 (Invitrogen) by digestion with PstI,
441 then cloned into DraI-digested pKR68, disrupting the β -lactamase gene. The resulting plasmid,
442 pKR128 pYES2 Tn7-*lacZ*, was used for subsequent cloning of 5' UTRs using alanine linkers
443 and NotI sites, as described above and detailed in **Table S3**. pYES2-based plasmids were
444 purified from overnight cultures of *S. cerevisiae* using the Zymoprep Yeast Plasmid Miniprep III
445 kit.

446 pF-GFP plasmids

447 Multicopy GFP reporter plasmids were created from a previously described shuttle
448 vector, pFNLTP6 (31). A fragment containing the promoter, 5' UTR, and first six codons of *tul4*
449 was digested from pKR89 with KpnI/NotI. sfGFP codon-optimized for expression in *F.*
450 *tularensis* LVS was purchased as a gBlock (IDT) and digested with NotI/BamHI. Fragment
451 were cloned into KpnI/BamHI-digested pF such that GFP was in-frame with the first six codons
452 of *tul4*, resulting in pKR145 (pF-*tul4* UTR-GFP). The plasmid pKR146 (pF-*pdpA* UTR-GFP)
453 was constructed similarly, after amplification from pKR74 of the *tul4* promoter and *pdpA* 5'
454 UTR and first six codons and digestion of the PCR product with Kpn/NotI. Subsequent
455 constructs were cloned into pKR145 to replace the *tul4* 5' UTR using the endogenously
456 encoded PacI site in the *tul4* promoter (**Table S3** for details). For genes in which a
457 transcription start site had not been annotated at the time of plasmid design, 100 nucleotides
458 upstream of the start codon were included as the 5' UTR (**Table S3** for details). Known
459 transcription start sites for *tul4*, *iglA*, and *pdpA* were previously published by Ramsey et al.
460 (32); the transcription start site for *hfq* was experimentally determined by Meibom et al. (21)
461 and Chambers & Bender (33).

462 pF-nat complementation vector

463 The pF-*rpsU2*-V plasmid (pKR7, 14) was modified to replace the kanamycin resistance
464 cassette with a nourseothricin (nat) resistance cassette from pF3-MglA-V as previously
465 described (34), yielding pKR15 pF-nat-*rpsU2*-V.

466 Allelic exchange plasmid

467 The plasmid pEX18kan was modified to create the in-frame deletion construct for
468 deletion of *hfq* as previously described (35). Flanking regions of ~1000 base pairs from both
469 sides of the *hfq* gene were amplified by PCR. Primers amplifying the DNA adjacent to *hfq*
470 included the first three or last three codons of the open reading frame and DNA specifying a
471 NotI site, which also encodes an alanine linker (5'-GCGGCCGCT-3'). The two fragments
472 were cloned into BamHI/KpnI-digested pEX18kan, yielding pKL111 pEXΔ*hfq*.

473 VSV-G tagging integration vector

474 A single integration vector for VSV-G tagging of *hfq* was made by modifying pKL02 (32).
475 The final 200 nucleotides of the 3' end of *hfq* was amplified using a 5' primer specifying a KpnI
476 site and a 3' primer that lacked the native stop codon and included DNA specifying a NotI site.
477 The fragment was cloned into KpnI/NotI-digested pKL02 such that the 3' end of *hfq* is in frame
478 with the codons specifying three alanine residues followed by the VSV-G epitope, resulting in
479 pKR158 (pEX-*hfq*-V).

480 **Strain construction**

481 β-galactosidase reporter strains (**Table 1**) were constructed by site- and orientation-
482 specific single chromosome integration using the Tn7 transposon as previously described (28).
483 Helper plasmid pMP720 was electroporated into either wild-type (LVS) or bS21-2 mutant
484 (Δ*rpsU2*) competent cells in 0.2 cm cuvettes with a 2.5 kV pulse and hygromycin-resistant cells

485 were selected by plating on CHA-H with hygromycin. Cells with the helper plasmid were
486 electroporated with the appropriate mini-Tn7 plasmid and selected for on CHA-H with
487 kanamycin. Colonies were screened for plasmid integration at the *attTn7* site using PCR.
488 Candidate strains were confirmed by amplification of genomic DNA outside of the *attTn7* site
489 and Sanger sequencing.

490 Reporter constructs encoded on pF plasmids were electroporated into LVS, LVS
491 $\Delta rpsU2$, or LVS Δhfq cells as described above and selected for on CHA-H with kanamycin
492 (**Table 1**). The complementation vectors or empty pF plasmids were electroporated into β -
493 galactosidase reporter strains as described above and selected for on CHA-H with
494 nourseothricin.

495 The Hfq deletion strain was constructed by allelic exchange as previously described
496 (14). Briefly, at least 1 μ g of allelic exchange plasmid pEX Δhfq was electroporated into
497 competent cells as above. Cells in which a single integration event occurred were selected for
498 on CHA-H-kanamycin. Counter-selection for the vector was accomplished by plating on CHA-
499 H (BD Difco) containing 10% sucrose. Sucrose-resistant, kanamycin-sensitive colonies were
500 screened for deletions using PCR. Candidate strains were confirmed by amplification of
501 genomic DNA outside of the flanking regions on each side of the deletion and Sanger
502 sequencing, validating LVS Δhfq .

503 Cells with VSV-G-tagged Hfq were made as previously described (32). Briefly, at least 1
504 μ g of pKR158 pEX-*hfq*-V was electroporated into LVS and $\Delta rpsU2$ cells and transformants
505 were selected on CHA-H-with kanamycin. Cells were confirmed to have a single integration by
506 PCR amplification of DNA across the integration site and subsequent Sanger sequencing of
507 the PCR product.

508 ***β-galactosidase assays***

509 *β*-galactosidase assays using *F. tularensis* LVS or *ΔrpsU2* cells containing indicated
510 reporter constructs were conducted as previously described (29). If significant yellow color was
511 not produced within two hours, reactions were stopped at 120 minutes. Experiments were
512 conducted at least twice in biological triplicate.

513 ***GFP assays***

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

521 ***Plasmid copy number qPCR***

522 Wild-type LVS or LVS *ΔrpsU2* cells with pF plasmids were grown to mid-log (OD₆₀₀ =
523 0.3-0.4). Total DNA was extracted from 1 mL culture using the MasterPure Complete DNA
524 purification kit (Lucigen). qRT-PCR was performed using PowerUp SYBR Green Master Mix
525 (Applied Biosystems) and a Roche LightCycler 480 (RI-INBRE CRCF) essentially as described
526 (35) with 0.05 ng of DNA. DNA abundances were calculated for an opening reading frame
527 (ORF3) on the plasmid and relative abundance is reported compared to a chromosomally-
528 encoded control gene, *tul4*. Experiments comparing wild-type and *rpsU2* mutant cells were
529 performed three times in biological triplicate.

530 **5' UTR secondary structure prediction**

531 Secondary structures reported above were predicted using the MXfold2 web server
532 (36).

533 **5' UTR motif analyses**

534 The 5' UTRs of genes that have decreases in protein, but not transcript, abundance in
535 cells lacking bS21-2 compared to wild-type (14) were analyzed. As there is insufficient
536 annotation of transcriptional start sites in *F. tularensis* LVS, 100 nucleotides upstream of the
537 start codon along with the first six codons were chosen for analysis.

538 STREME software (MEME suite) was used to analyze the 5' UTRs of the 20 genes with
539 the largest fold decreases in protein in $\Delta rpsU2$ cells compared to LVS. These were compared
540 to shuffled sequences to find two candidate motifs. As a control, the same parameters were
541 used to compare the predicted 5' UTRs of 20 genes not differentially expressed in LVS and
542 $\Delta rpsU2$.

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

545 **Immunoblotting**

546 Immunoblotting was completed as previously described (14). Briefly, cell lysates were
547 separated by SDS-PAGE, transferred to PVDF, and analyzed for total protein with the
548 Invitrogen No-Stain Protein labeling reagent for normalization. Membranes were probed with
549 indicated monoclonal antibodies (BEI Resources, diluted 1:250 for PdpB, 1:1000 for IgIB, and
550 1:1000 for IgIA) or the VSV-G epitope tag (Sigma, diluted 1:2222) in blocking buffer. Proteins
551 were detected using IRDye 800 CW donkey anti-mouse IgG or donkey anti-rabbit IgG
552 secondary antibodies (Li-Cor, diluted 1:10,000). Protein abundance was calculated as

553 fluorescence of protein bands relative to total protein in each lane. Experiments were
554 performed at least twice in biological triplicate.

555 ***RNA purification and qRT-PCR***

556 Total RNA was purified according to the RNAsnapTM protocol (37). *F. tularensis* LVS
557 was grown in biological triplicate to mid-log phase. Pelleted cells (10 mL) were resuspended in
558 100 µl of fresh RES (95% formamide, 18 mM EDTA, 0.025% SDS, 1% BME), then incubated
559 at 95°C for 7 minutes. Cell debris were pelleted by centrifugation and the supernatant was
560 preserved. Total nucleic acid was recovered with 0.3M sodium acetate (pH 5.2) and 3x
561 volumes 100% ethanol. Samples were stored at -80°C for 1 hour, then nucleic acid was
562 pelleted by centrifugation at 4°C for 30 minutes. The pellet was washed with 75% ethanol and
563 resuspended in water. Purified nucleic acids were treated with RQ1 DNase (Promega) for 1
564 hour at 37°C and RNA was purified again with sodium acetate/ethanol precipitation.

565 cDNA was synthesized using Superscript III reverse transcriptase (Life Technologies)
566 as previously described (35). qRT-PCR was performed with the PowerUP SYBR Green Master
567 Mix (Applied Biosystems) and the Roche Lightcycler 480 (RI-INBRE CRCF). Transcript
568 abundances of *pdpA*, *pdpB*, *iglA*, *pigR*, *rpoA1*, and *bfr* were normalized to a control gene, *tul4*.
569 Experiments were conducted twice in biological triplicate.

570

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592

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719 **Table 1: Strains used in this study**

| Strain Number | Description | Genetic Background | Plasmid |
|--|---|--------------------|---------|
| β-galactosidase reporter strains | | | |
| KRLVS96 | LVS Tn7::Ptul4-pdpA 5'UTR-lacZ aphA | LVS | pKR74 |
| KRLVS97 | LVS $\Delta rpsU2$ Tn7::Ptul4-pdpA 5'UTR-lacZ aphA | $\Delta rpsU2$ | pKR74 |
| KRLVS102 | LVS Tn7::Ptul4-pdpA 5'UTR-mut1-lacZ aphA | LVS | pKR84 |
| KRLVS106 | LVS $\Delta rpsU2$ Tn7::Ptul4-pdpA 5'UTR-mut1-lacZ aphA | $\Delta rpsU2$ | pKR84 |
| KRLVS103 | LVS Tn7::Ptul4-pdpA 5'UTR-mut2-lacZ aphA | LVS | pKR86 |
| KRLVS108 | LVS $\Delta rpsU2$ Tn7::Ptul4-pdpA 5'UTR-mut2-lacZ aphA | $\Delta rpsU2$ | pKR86 |
| KRLVS114 | LVS Tn7::Ptul4-pdpA 5'UTR-badSD-lacZ aphA | LVS | pKR98 |
| KRLVS117 | LVS $\Delta rpsU2$ Tn7::Ptul4-pdpA 5'UTR-badSD-lacZ aphA | $\Delta rpsU2$ | pKR98 |
| KRLVS115 | LVS Tn7::Ptul4-pdpA 5'UTR-ideal movedSD-lacZ aphA | LVS | pKR99 |
| KRLVS118 | LVS $\Delta rpsU2$ Tn7::Ptul4-pdpA 5'UTR-ideal movedSD-lacZ aphA | $\Delta rpsU2$ | pKR99 |
| KRLVS112 | LVS Tn7::Ptul4-tul4 5'UTR-lacZ aphA | LVS | pKR89 |
| KRLVS111 | LVS $\Delta rpsU2$ Tn7::Ptul4-tul4 5'UTR-lacZ aphA | $\Delta rpsU2$ | pKR89 |
| KRLVS158 | LVS Tn7::Ptul4-pdpA 5'UTR-idealSD-lacZ aphA | LVS | pKR129 |
| KRLVS160 | LVS $\Delta rpsU2$ Tn7::Ptul4-pdpA 5'UTR-idealSD-lacZ aphA | $\Delta rpsU2$ | pKR129 |
| KRLVS159 | LVS Tn7::Ptul4-pdpA 5'UTR-tul4SD-lacZ aphA | LVS | pKR130 |
| KRLVS161 | LVS $\Delta rpsU2$ Tn7::Ptul4-pdpA 5'UTR-tul4SD-lacZ aphA | $\Delta rpsU2$ | pKR130 |
| KRLVS266 | LVS Tn7::Ptul4-pdpA 5'UTR-lacZ aphA pF-nat | KRLVS96 | pF-nat |
| KRLVS267 | LVS $\Delta rpsU2$ Tn7::Ptul4-pdpA 5'UTR-lacZ aphA pF-nat | KRLVS97 | pF-nat |
| KRLVS268 | LVS $\Delta rpsU2$ Tn7::Ptul4-pdpA 5'UTR-lacZ aphA pF-nat-rpsU2-V | KRLVS97 | pKR15 |
| KRLVS269 | LVS Tn7::Ptul4-tul4 5'UTR-lacZ aphA pF-nat | KRLVS112 | pF-nat |
| KRLVS270 | LVS $\Delta rpsU2$ Tn7::Ptul4-tul4 5'UTR-lacZ aphA pF-nat | KRLVS111 | pF-nat |
| pF-GFP reporter strains | | | |
| KRLVS180 | LVS pF-tul4UTR-GFP | LVS | pKR145 |
| KRLVS182 | LVS $\Delta rpsU2$ pF-tul4UTR-GFP | $\Delta rpsU2$ | pKR145 |
| KRLVS234 | LVS Δhfq pF-tul4 UTR-GFP | Δhfq | pKR145 |
| KRLVS181 | LVS pF-pdpAUTR-GFP | LVS | pKR146 |
| KRLVS183 | LVS $\Delta rpsU2$ pF-pdpAUTR-GFP | $\Delta rpsU2$ | pKR146 |
| KRLVS236 | LVS Δhfq pF-pdpA UTR-GFP | Δhfq | pKR146 |
| KRLVS188 | LVS pF-mraYUTR-GFP | LVS | pKR151 |
| KRLVS189 | LVS $\Delta rpsU2$ pF-mraYUTR-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-mraYUTR_mut1-GFP | LVS | pKR156 |
| KRLVS200 | LVS $\Delta rpsU2$ pF-mraYUTR_mut1-GFP | $\Delta rpsU2$ | pKR156 |
| KRLVS201 | LVS pF-mraYUTR_mut2-GFP | LVS | pKR157 |
| KRLVS202 | LVS $\Delta rpsU2$ pF-mraYUTR_mut2-GFP | $\Delta rpsU2$ | pKR157 |
| KRLVS206 | LVS pF-iglAUTR-GFP | LVS | pKR160 |
| KRLVS207 | LVS $\Delta rpsU2$ pF-iglAUTR-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-mraYUTR_mut5-GFP | LVS | pKR165 |
| KRLVS215 | LVS $\Delta rpsU2$ pF-mraYUTR_mut5-GFP | $\Delta rpsU2$ | pKR165 |
| KRLVS222 | LVS pF-mraYUTR_idealSD-GFP | LVS | pKR169 |
| KRLVS223 | LVS $\Delta rpsU2$ pF-mraYUTR_idealSD-GFP | $\Delta rpsU2$ | pKR169 |
| KRLVS228 | LVS pF-hfqUTR-GFP | LVS | pKR172 |
| KRLVS229 | LVS $\Delta rpsU2$ pF-hfqUTR-GFP | $\Delta rpsU2$ | pKR172 |
| KRLVS243 | LVS pF-mraYUTR_mut6-GFP | LVS | pKR175 |
| KRLVS244 | LVS $\Delta rpsU2$ pF-mraYUTR_mut6-GFP | $\Delta rpsU2$ | pKR175 |
| KRLVS247 | LVS pF-mraYUTR_mut7-GFP | LVS | pKR177 |
| KRLVS248 | LVS $\Delta rpsU2$ pF-mraYUTR_mut7-GFP | $\Delta rpsU2$ | pKR177 |

| Strain Number | Description | Genetic Background | Plasmid |
|-----------------------------|--|--------------------|---------|
| KRLVS252 | LVS pF- <i>mraY</i> UTR_mut8-GFP | LVS | pKR179 |
| KRLVS253 | LVS $\Delta rpsU2$ pF- <i>mraY</i> UTR_mut8-GFP | $\Delta rpsU2$ | pKR179 |
| KRLVS260 | LVS pF- <i>mraY</i> UTR_mut9-GFP | LVS | pKR180 |
| KRLVS261 | LVS $\Delta rpsU2$ pF- <i>mraY</i> UTR_mut9-GFP | $\Delta rpsU2$ | pKR180 |
| KRLVS262 | LVS pF- <i>mraY</i> UTR_mut3-GFP | LVS | pKR182 |
| KRLVS263 | LVS $\Delta rpsU2$ pF- <i>mraY</i> UTR_mut3-GFP | $\Delta rpsU2$ | pKR182 |
| KRLVS264 | LVS pF- <i>mraY</i> UTR_mut4-GFP | LVS | pKR183 |
| KRLVS265 | LVS $\Delta rpsU2$ pF- <i>mraY</i> UTR_mut4-GFP | $\Delta rpsU2$ | pKR183 |
| KRLVS197 | LVS pF- <i>mraY</i> UTR_mut10-GFP | LVS | pKR155 |
| KRLVS198 | LVS $\Delta rpsU2$ pF- <i>mraY</i> UTR_mut10-GFP | $\Delta rpsU2$ | pKR155 |
| 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 |

Figure Legends

Figure 1. 5' UTRs are sufficient to lead to bS21-2-mediated changes in translation. (A)

Diagram of the translational reporter fusions used. Reporters used the *tul4* promoter to drive production of mRNA including the tested 5' UTR with the first 6 codons of the gene. Leader sequences are in frame with either *lacZ* (integrated at the Tn7 site of the genome) or *gfp* (on a plasmid). (B) Relative fluorescence for indicated *gfp* translational fusion reporters in cells with (+; WT) or without (-; $\Delta rpsU2$) bS21-2. *tul4* serves as a control. 5' UTR sequences can be found in Supplemental Table 1. (C) Relative β -galactosidase activity for indicated *lacZ* translational fusions in cells with (+; WT) or without (-; $\Delta rpsU2$) native bS21-2, or with ectopically expressed bS21-2 from a plasmid (pF-bS21-2). Strains without ectopically expressed bS21-2 contained an empty vector (pF). (B and C) Error bars represent 1 SD. Lines above bars indicate comparisons, values above line indicate ratio of reporter activity in cells lacking bS21-2 to wild-type cells. * $p < 0.05$. ns = not significant. Experiments were repeated at least twice in biological triplicate and data from a representative experiment are shown.

Figure 2. Genes with ideal Shine-Dalgarno (SD) sequences are not responsive to bS21-2.

(A) bS21 interacts with the anti-Shine Dalgarno (ASD) sequence. In *E. coli*, amino acid R17 of the sole bS21 protein (green) directly interacts with C1539 of 16S rRNA, which is part of the ASD (blue). Measured distance is 2.7Å (PDB 6o7k; 15). (B) The absence of strong SD-ASD interactions is correlated with bS21-2 influencing translation. Fraction of genes that are positively-impacted (n=74), negatively-impacted (n=84), or unaffected (n=82) by bS21-2, categorized by strength of SD. "Strong" SD: 4 or more nts complementary to ASD; "weak" SD: 3 or fewer complementary nts. (C) Introduction of an ideal SD in the *pdpA* leader leads to loss

745 of bS21-2 responsiveness. Top: Relative β -galactosidase activity for indicated *lacZ*
746 translational fusions in cells with (+; WT) or without (-; $\Delta rpsU2$) bS21-2. Lines above bars
747 indicate comparisons, values above line indicate ratio of reporter activity in cells lacking bS21-
748 2 to wild-type cells. Error bars represent 1 SD. * $p < 0.05$ by t-test. ns=not significant.
749 Experiments were repeated at least twice in biological triplicate and data from a representative
750 experiment are shown. Bottom: Alignment of modifications to *pdpA* 5' UTR. Capital letters:
751 altered from WT; bold and underlined: predicted SD sequences; unnormalized β -galactosidase
752 activity can be found in Fig S3.

753

754 **Figure 3. bS21-2-mediated translation of *mraY* depends on a weak SD and a specific 6 nt**
755 **sequence.** Charts show relative fluorescence for indicated *gfp* translation fusion reporters in
756 cells with (+; WT) or without (-; $\Delta rpsU2$) bS21-2. **(A)** Introduction of an ideal SD in the *mraY*
757 leader leads to loss of bS21-2 responsiveness. **(B)** A motif common in bS21-2 responsive
758 leader sequences (Motif 1, see F and Fig S5) is not necessary for bS21-2-responsive
759 translation in the *mraY* 5' UTR. A truncation that removes both motifs enriched in bS21-2
760 responsive leaders is no longer responsive (mut10). **(C)** The nucleotides between -58 – -63 in
761 the *mraY* 5' UTR, GACUCU, are essential for responsiveness to bS21-2. **(D)** Secondary
762 structure predictions of wild-type and modified *mraY* 5' UTRs. The 6 nt sequence necessary
763 for responsiveness to bS21-2 is highlighted in yellow in *mraY* and *mraY* mut3 structures and
764 the location of mutated residues is highlighted in grey in *mraY* mut4. **(E)** Changing the *mraY* 5'
765 UTR secondary structure does not impact responsiveness to bS21-2. **(A-C, E)** Lines above
766 bars indicate comparisons, values above line indicate ratio of reporter activity in cells lacking
767 bS21-2 to wild-type cells. Error bars represent 1 SD. * $p < 0.05$ by t-test. ns=not significant.

768 Experiments were repeated at least twice in biological triplicate and data from a representative
769 experiment are shown. Unnormalized fluorescence values can be found in Fig S4. **(F)**
770 Alignment of tested modifications to the *mraY* 5' UTR. Sequences are grouped according to
771 the panel in which they are found. Modifications from the wild-type leader sequence are
772 capitalized and bold. Predicted and modified SD sequences are underlined in the sequences
773 corresponding to panel A. Sequences corresponding to motifs enriched in bS21-2 responsive
774 leaders are indicated. The key 6 nt sequence necessary for responsiveness to bS21-2 is found
775 at nt -58 – -63 and is highlighted in yellow.

776

777 **Figure 4. Hfq and bS21-2 influence production of T6SS proteins via different pathways.**

778 **(A)** Diagram of the Francisella Pathogenicity Island (FPI) genes, which encode T6SS proteins.
779 **(B)** Cells lacking bS21-2 have more Hfq. Bottom: Immunoblots probed with anti-VSV-G
780 antibody. Whole cell lysates from bacteria containing Hfq-VSV-G and either with (WT) or
781 without ($\Delta rpsU2$) bS21-2. Top: Quantification of immunoblots. Band intensities for each protein
782 were normalized to total protein per lane on the membrane. **(C)** Loss of bS21-2 leads to more
783 *hfq* translation. Relative fluorescence for indicated *gfp* translational fusion reporters in cells
784 with (+; WT) or without (-; $\Delta rpsU2$) bS21-2. **(D)** Only some of the T6SS proteins are influenced
785 by loss of Hfq. Bottom: Immunoblots probed with antibodies to indicated T6SS proteins in
786 lysates of WT cells, cells lacking bS21-2 ($\Delta rpsU2$), or cells lacking Hfq (Δhfq). Top:
787 Quantification of immunoblots. Band intensities for each protein were normalized to total
788 protein per lane on the membrane. **(E)** Hfq does not influence translation of the T6SS protein
789 PdpA. Relative fluorescence for indicated *gfp* translational fusion reporters in WT cells, cells
790 lacking bS21-2 ($\Delta rpsU2$), or cells lacking Hfq (Δhfq). **(F)** Hfq is a negative regulator of

791 transcript abundance for some T6SS genes. Quantitative RT-PCR was used to determine the
792 relative transcript abundance for indicated FPI-encoded genes in WT cells, cells lacking bS21-
793 2 ($\Delta rpsU2$), or cells lacking Hfq (Δhfq), normalized to the *tul4* gene. The *rpoA1* and *bfr* genes
794 are included as additional negative controls, as their expression is not meaningfully influenced
795 by bS21-2. **(B-F)** Lines above bars indicate comparisons, values above line indicate ratio of
796 reporter activity in cells lacking bS21-2 to wild-type cells. Error bars represent 1 SD.
797 Experiments were repeated at least twice in biological triplicate and data from a representative
798 experiment are shown. **(A-D)** * $p < 0.05$ after Bonferroni correction. **(F)** * $p < 0.005$ after
799 Bonferroni correction, compared to WT.

800







