

Point-by-point response to reviewer comments

The reviewer comments are in **blue** and our responses are in **black**.

Reviewer #1

This paper investigates the action of the *Francisella tularensis* ribosome protein bS21-2, which is one homolog of bS21 and whose deletion results in a changed proteome. The authors show that 1) being responsive to bS21-2 correlates to sequences within the 5'UTR and with a weaker Shine-Dalgarno sequence, 2) for one of the affected proteins, *mraY*, the deletion of a hexamer sequence 58 nt upstream of the AUG start, eliminates the bS21-2 effect, and 3) for certain bS21-2-responsive T6SS genes, the RNA chaperone Hfq acts to decrease transcript abundance, while bS21-2 works to increase translation. The work has been performed well. These are interesting findings that would be interesting to those in the translation field and to those investigating regulation of bacterial virulence.

My main concern with the paper is its overall organization and presentation of the data, because as written, I believe that it is quite difficult for the reader to follow. Consequently, these findings are not as impactful as they should be.

I suggest the following:

1. Start with a discussion of the previously found bS21-2 proteome and the analyses in Fig. 2 and 3D, showing that the 5' UTR and, in general, a weaker SD site correlates with bS21-2 responsiveness.
2. Show the full analysis of *mraY* combining the info in Fig. 3C, 4, 5B, and 6 to show what is not and what is needed for bS21-2 responsiveness with this gene. This would then combine the complete mutational analysis of *mraY* in one section. Please highlight the GACUCU sequence throughout these figures, both in the sequence and in the secondary structures. Please add an analysis of whether this site is present in the other bS21-2 responsive genes (apparently it is not) and show the sequences of the other genes in a supplemental table.
3. Show the analysis of the T6SS genes, and the conclusion that Hfq and bS21-2 work in different ways.

I believe that this organization would make the paper much easier to read and would greatly enhance its impact.

Response 1

We agree with the reviewer that re-organization may better demonstrate the key findings. We have modified the structure of the paper and order of the figures per their recommendations, including a complete mutational analysis of *mraY* in one figure, Figure 3. It should be noted that due to this reorganization, we included data from a translational reporter of a modified *pdpA* leader sequence that was previously omitted (*pdpA* mut10). While data from this translational reporter do not alter the key points (thus its omission from the original submission), we believe that including these data improves the current version of the paper. We have also included a

new supplemental table that provides all of the 5' UTR sequences of genes positively impacted by bS21-2 at the protein level (Table S1). We appreciate the thoughtful and helpful suggestion and believe this re-organization has significantly improved the manuscript.

Other comments:

Line 138 should read 'on both operons'

Response 2

We have made this change (line 295).

Line 161 - Increase looks more like 10-fold and 23 -fold? Please explain.

Response 3

Thank you for noting this error. We repeated this experiment multiple times and the current figure references a representative experiment; the original version of the text referenced a different replicate. We have updated the text to reflect the appropriate values (line 311).

Fig. 1. Please make the bar colors consistent throughout the panels (same color for same gene)

Response 4

As suggested for former Figure 1 (now Figure 4), we have modified the colors such that one color represents information from one gene.

In Fig. 1E, please show from 0 to 10 as most of the graph and then have a double line to indicate the higher 25 value.

Response 5

We appreciate that the reviewer would like to evaluate the smaller changes in relative transcript abundance indicated by this chart. However, the intent of the chart is to demonstrate the large changes in transcript abundance for only two genes in cells lacking Hfq, compared to the modest changes for other transcripts. As such, we think the data presented this way are most impactful. However, to address this important concern, we have added Table S2, which reports all the values depicted in this figure.

Please move Fig. S3 to the main text as this is important.

Response 6

We have moved former Fig. S3 to the main text (Figure 1C).

Line 213 should read Fig. 2B?

Response 7

While this reference was not retained during the reorganization of the text, we have updated all figure number references and ensured that they are correct.

Line 217 says C1529 while Fig. 3A says C1539.

Response 8

We have updated this typographical error (C1539 is correct, line 134).

Fig. 3B What sequence in *pdpA* do you consider to be the SD? Is it the AAGUAG that is too close to the AUG or the AUUAAG that is in the correct position? Please mark in 3B and in 3C. Also please indicate the absolute b-gal units in the legend. This is important since the 'BadSD' probably gives very low units.

Response 9

We appreciate the reviewer's suggestion to indicate the predicted Shine Dalgarno for *pdpA* and have now included this in Figure 2 (and for the *mraY* 5' UTR in Figure 3). As described in the methods, "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." As such, our predicted SD for *pdpA* is AGUAGA, which is indeed in an unlikely position. However, when constructing the "badSD" leader sequence, we altered both regions that the reviewer has suggested as potential SDs in order to minimize SD-ASD binding at either location.

We also agree with the reviewer that the absolute β -galactosidase units are relevant. Rather than include this information in the legend, we have decided to add supplemental figures to show all of the unnormalized values for modified leader sequences. Specifically, Fig 2 and 3 report normalized values and Figs S3 and S4 report unnormalized values, respectively. We have also added text to the results and conclusions addressing the relative amount of reporter activity produced from modified leader sequences and responsiveness to bS21-2. In particular, lines 230 – 233 address how modifications to the *mraY* leader influence total protein synthesis and lines 359– 369 address a similar issue in the context of the *pdpA* leader with altered Shine Dalgarno sequences.

Line 275 I did not see a Fig. S5.

Response 10

We have altered and updated the text to remove this incorrect reference (line 239).

Discussion. Much of the Discussion is a recapitulation of the results. I would shorten this and then discuss what is known about bS21-2 in other systems and how and if your findings relate to this.

Response 11

We appreciate the reviewer's recommendations to modify this section and have removed text that was redundant with the Results section. Unfortunately, as discussed in the introduction, there is limited research into bS21 in other species outside of the Bacteroidia, and the bS21 homologs in Bacteroidia are likely structurally and functionally distinct from bS21 homologs in

other phyla. Given our focus on the sequence-dependent nature of regulation by bS21-2, we added a brief discussion of how this might extend to other organisms (lines 380-386).

Reviewer #2:

Review of Trautmann et al 2023

Here Trautmann and colleagues have investigated the mechanisms by which the ribosomal protein bS21-2 affects expression of T6SS genes in *Francisella tularensis*. They found that 5' UTRs from some of the affected genes were sufficient to make reporter genes affected by bS21-2. The 5' UTR features that produce this effect appear to be complex. The effect was abrogated by optimization of the SD sequence, suggesting that bS21-2 promotes translation of genes with weak SD sequences. Overall, the study is well done and well presented and will be a useful contribution to the field.

My comments are all minor, as follows:

It would be helpful to include a schematic of the two T6SS operons in the first figure to make it easy to see which of the genes tested in various places in the paper belong to which operon.

Response 12

We agree with the reviewer's recommendation and have added a diagram showing the *Francisella* Pathogenicity Island, encoding the T6SS, as Figure 4A.

Fig 1: Please put the panels in order A-E to improve readability.

Response 13

This figure (now Figure 4) has been updated for improved readability.

Fig 3 and others that show beta-gal activity or fluorescence: Please show the data as arbitrary units, not normalized to the WT version of each gene. The expression levels of the constructs may vary, and sometimes this is important, as is shown in one of the supplementary figures with respect to the different impact of bS21-2 for constructs with strong SD sequences. Rather than put this in the supplement, it would be cleaner and more informative to omit the normalization from all the figures and thus retain data that may be interesting and important.

Response 14

We appreciate the reviewer's recommendation to show unnormalized values for the B-galactosidase and GFP assays. While we agree that these values are informative, the focus of this study is on the relative change in reporter activity in the cells lacking bS21-2 compared to wild-type cells, so we have retained the normalized values in the main figures. Maintaining the normalized values also improves readability as data from each 5' UTR are on the same scale. However, to address this important concern, we have included unnormalized values for all the translational reporter assays testing modified leader sequences. Specifically, normalized data are in Figs 2 and 3 while unnormalized data can be found in Figs S3 and S4. Fig S6 includes both

normalized and unnormalized values. As noted in Response 9, we have also included text addressing relative amounts reporter protein synthesized from modified leaders.

Fig 5: Please make the secondary structure figures much larger so the numbers and bases can be read.

Response 15

The images for the secondary structures have been increased in size (now Figure 3D and Figure S6).

Fig 6: Please add shading or dashed lines connecting the allele names to the sequence to make it easier for the eye to follow.

Response 16

We have added light grey shading to the sequences to improve readability in Figures 2, 3, and S6.

Fig S1: Change the word "opening" in the legend to "open."

Response 17

We have corrected this typographical error in the Supplemental Figures Legend document.

Fig S3: The figure legends state that fluorescence readings were normalized to a non-fluorescent strain. However, the methods section states that readings from non-fluorescent strains were subtracted (which to this reviewer makes more sense). Please correct the language as needed.

Response 18

We have corrected the language in the figure legends to indicate that readings from a non-fluorescent strain were subtracted, not normalized, in the Supplemental Figures Legend document.

Line 12, it is unclear what is meant by the term "in contrast." In contrast to what? I suggest revising the sentence.

Response 19

This sentence has been rewritten to remove the unclear phrasing (line 10).

Line 29, replace "is" with "are."

Response 20

This typographical error has been corrected (line 29).

Line 55, the word "in" is missing.

Response 21

This typographical error has been corrected (line 51).

Line 60, "mRNA" should be singular.

Response 22

This typographical error has been corrected (line 56).

Line 68, I suggest putting the reference at the end of this sentence rather than at the end of the paragraph.

Response 23

We have made this modification to move the location of the reference (line 64).

Line 101, replace the word "genes" with the word "transcripts," assuming that is what is meant.

Response 24

This sentence was removed due to the reorganization of the text.

Line 103, it is confusing whether the effect being referred to is a transcript abundance effect, a protein abundance effect, or both.

Response 25

We have modified the phrasing to clarify our hypothesis – that there may be an effect on translation of a regulator, Hfq (sentence on lines 254-256).

Lines 114-115, was the tag integrated in the chromosome at the native locus? Specify this (or what was done if something different) to help the reader understand that the native regulatory sequences were controlling the tagged copy of the gene.

Response 26

We have added a clarification that the tag was added to the native locus of *hfq* on the chromosome (line 268).