

I. Background and key challenges

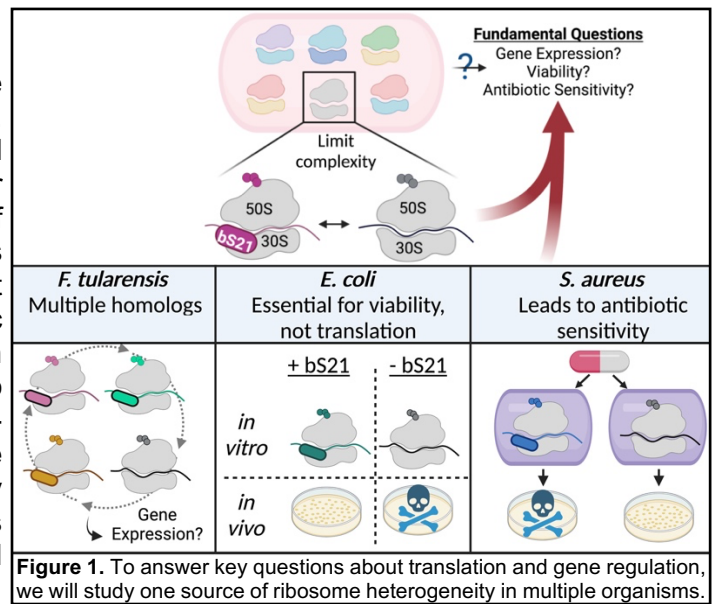
As antibiotic resistance increases globally, there is an urgent need to identify new antimicrobials¹. Successful novel antimicrobials could target essential proteins, proteins essential for a specific pathogen, or proteins critical for bacterial virulence. A major benefit of identifying pathogen- or virulence-specific processes and drugs that target them would be a reduced impact on the commensal host microbiome². By studying basic mechanisms of bacterial gene expression, we can both better understand the essential processes that lead to gene regulation and identify new, potentially species-specific, targets for antimicrobial development. **The overarching focus of the research program in my laboratory is to understand how bacterial cells regulate gene expression, to ultimately identify novel targets for antimicrobial development.**

The ribosome is an essential player in gene expression and a major drug target; a large number of ribosome-inhibiting antibiotics are used clinically. Extensive research in ribosome biology has led to major advances in our understanding of translation and ribosome-targeting drugs^{3–5}. Most studies have focused on model organisms like *Escherichia coli* and fewer have examined ribosomes in other pathogenic bacteria. While the mechanism of protein synthesis is conserved across all domains of life, there are significant differences in ribosome composition among bacterial species. Even within the same species, there can be significant heterogeneity in ribosome composition⁶. Ribosomal proteins (r-proteins) provide one source of this heterogeneity. R-protein sequences can differ significantly between species and even in the same organism; most bacteria encode more than one homolog for at least one r-protein⁷. The functional consequences of bacterial ribosome heterogeneity have yet to be fully understood and there are many open questions in this field⁸. This is particularly true with respect to ribosomes containing alternate r-proteins in the same bacterial species^{9–12}. **The impacts of ribosome heterogeneity on translation, gene expression, and drug efficacy are generally not well understood and represent key gaps in our knowledge.**

There are some well-understood examples of how bacterial ribosomes become heterogenous. While it is extremely clear how changes in the environment can lead to regulated changes in ribosome composition, it is unclear how or if these changes result in modified ribosome activity and/or function. One well-studied example of ribosome heterogeneity is the incorporation of r-protein paralogs that either do or do not coordinate zinc^{10,11,13–17}. In some organisms, including mycobacteria and *Bacillus subtilis*, r-protein paralogs capable of coordinating zinc are produced and incorporated into ribosomes in zinc-replete conditions. When zinc levels are lower, a coordinated program of gene expression leads to production of multiple r-protein paralogs that do not coordinate zinc, which are subsequently incorporated into the ribosome. This paralog switching appears to allow dynamic control of available zinc inside the cell but the consequences of this heterogeneity on ribosome function are less clear. One complication is that this switch involves multiple r-proteins (four in *Mycobacterium tuberculosis*, for example), so ribosomes with many different r-protein combinations may be present in each cell. Nonetheless, evidence is accumulating that these alternate ribosomes do lead to changes in gene expression, although the molecular mechanisms have yet to be elucidated^{9,12}.

We propose to answer key questions about the impact of ribosome heterogeneity on gene expression, cell survival, and antimicrobial sensitivity by taking an innovative strategy and focusing on one source of ribosome heterogeneity, the r-protein bS21, in multiple bacterial species (Figure 1). The proposed work is innovative because it takes a rigorous, reductionist approach to examine one poorly-understood source of ribosome heterogeneity with species-specific impacts on translation to elucidate fundamental features of translation and inform the design of novel antimicrobials.

Our laboratory has recently identified a previously uncharacterized source of bacterial ribosome heterogeneity (unrelated to zinc) that impacts gene expression and antibiotic sensitivity. This heterogeneity is due to incorporation of distinct homologs of the r-protein bS21 into *Francisella tularensis* ribosomes¹⁸ (described in detail in Section II, Recent Progress; bS21 was previously known as S21¹⁹). While it is thought to play a role in translation initiation^{20,21}, **the precise function of bS21 in translation is not known.** This small subunit r-



protein, in contrast to many other proteins, is ideally located in the ribosome to function in a regulatory manner. It is found next to the mRNA exit channel, a position in which it can interact with the 5' untranslated region (5' UTR) of transcripts during translation initiation, and makes contacts with the anti-Shine-Dalgarno sequence of the 16S rRNA (the sequence that base-pairs with the Shine-Dalgarno sequence of mRNAs, facilitating ribosome binding to the translation start site)²²⁻²⁴. As one of the last r-proteins to be incorporated, it is not essential for ribosome assembly²⁵. In fact, because bS21 has been lost in multiple lineages across the bacterial phylogeny, it is thought to be non-essential to translation^{7,26,27}. Finally, bS21 is considered loosely-associated with the ribosome and can be easily exchanged, suggesting that in organisms that encode multiple bS21 homologs ribosomes may rapidly switch between homologs^{28,29}. **These characteristics suggest that, instead of being essential for ribosome assembly or function, bS21 plays a regulatory role during translation initiation.**

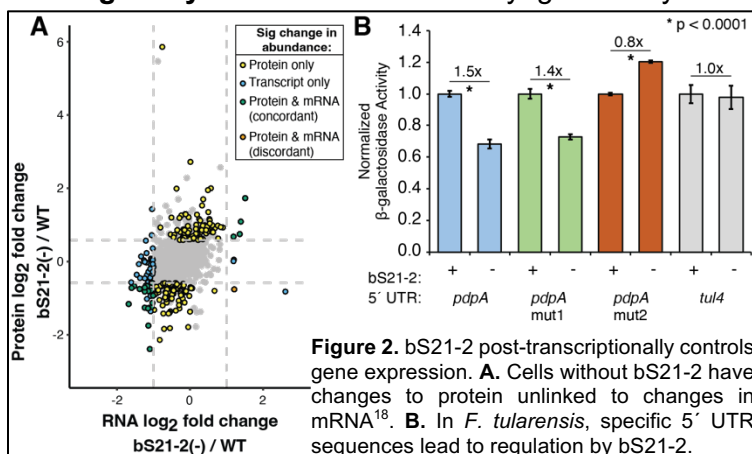
Multiple observations support the idea that bS21 may function to regulate translation. For example, bS21 in *E. coli* is important for translation of some, but not all, transcripts²¹. In other species, cells with bS21 mutations exhibit distinct phenotypes including reduced motility and biofilm formation, altered stress resistance, increased resistance to antibiotics, and decreased virulence³⁰⁻³⁹. A recent study of the ribosomes from Bacteroidetes revealed that bS21 homologs in these organisms have distinct C-terminal regions that, in a representative organism (*Flavobacterium johnsoniae*), interact directly with and occlude, the anti-Shine-Dalgarno sequence⁴⁰. While most Bacteroidetes mRNAs lack a Shine-Dalgarno sequence, the transcript encoding bS21 contains a consensus Shine-Dalgarno sequence, suggesting a straightforward mechanism for bS21 to control its own production⁴⁰. Finally, consistent with the potential for different bS21 homologs to influence gene expression, multiple bacteriophage-encoded bS21 genes have been identified and at least one bacteriophage-encoded bS21 can be incorporated into *E. coli* ribosomes⁴¹⁻⁴³. This raises the possibility that bacteriophage may use bS21 homologs in their reprogramming of infected cells. **While these diverse phenotypes can all be linked to bS21, it is not known how bS21 exerts its effect at the molecular level.** These findings suggest that bS21 is important for regulating specific pathways, possibly by regulating translation initiation.

While evidence suggests that bS21 may generally function as a regulator of translation, there appear to be organism-specific differences in the role of bS21. For example, in *F. johnsoniae*, bS21 may only function to control its own production while in *F. tularensis*, loss of one homolog changes the abundance of over 150 proteins. And while bS21 is not essential in some organisms, it appears to be an essential protein in others. Why this protein should be essential in some organisms but not others remains unclear. **By studying this single r-protein in multiple organisms, we will gain critical insights into ribosome function, ribosome heterogeneity, and the potential development of ribosome-specific antibiotics.**

II. Recent progress by the Ramsey laboratory

bS21 homologs provide a previously-unappreciated source of ribosome heterogeneity

In contrast to genomes encoding one or no bS21 homologs, the genomes of at least 50 bacteria contain two to four distinct genes encoding bS21 (*rpsU* genes). Among these species is the human pathogen *Francisella tularensis*. This organism is notable for having a small genome (<2 Mbps) which encodes relatively few transcription factors. Despite this small genome, *F. tularensis* contains three distinct genes encoding bS21, raising the possibility that ribosomes might incorporate different bS21 homologs and that this heterogeneity provides a post-transcriptional mechanism to regulate gene expression. This is the only apparent source of ribosome heterogeneity in *F. tularensis*, as all three rRNA operons are identical and there is only one gene encoding each of the other r-proteins. **We have demonstrated that distinct bS21 homologs lead to ribosome heterogeneity in *F. tularensis***¹⁸. By genetically manipulating *F. tularensis*, performing sucrose gradient



analyses, and through mass spectrometry analyses, we found that each bS21 homolog can be incorporated into *F. tularensis* ribosomes and that populations of wild-type ribosomes contain more than one bS21 homolog¹⁸.

A bS21 homolog governs gene expression, including key virulence genes

If bS21 homologs impact translation, we reasoned that their loss should result in changes to the proteome (but not the transcriptome). When we compared the proteome of cells lacking one homolog, bS21-2, to the transcriptome, we found changes in protein abundance for about 160 genes

that cannot be explained by changes in transcript abundance (**Figure 2A**, yellow dots)¹⁸. These changes include reduced abundance of type 6 secretion system (T6SS) proteins critical for *F. tularensis* virulence¹⁸. The abundance of the T6SS proteins can be complemented by ectopic expression of bS21-2 or another homolog, bS21-3¹⁸. Yet bS21-2 is uniquely important for intramacrophage survival of *F. tularensis*, suggesting that bS21-2 alone regulates additional virulence genes¹⁸. Our data are consistent with a model in which bS21 homologs modulate translation initiation for a subset of transcripts. Our successful use of unbiased multi-omics approaches revealed genome-wide changes in cells lacking bS21-2 and provides key information for future experiments.

5' UTR sequences can lead to regulation by ribosomes with bS21-2

We hypothesize that incorporation of specific bS21 homologs into the ribosome may lead to preferential translation initiation of a subset of transcripts. If this is the case, we further hypothesize that this regulation would be due to specific sequence elements in the 5' UTR of the transcript, as bS21 is located in an ideal location to interact with 5' UTRs. To test this possibility, we created *F. tularensis* reporter strains which use a constitutive promoter driving expression of different 5' UTRs fused to *lacZ* (encoding beta-galactosidase). For control 5' UTRs (genes unaffected by the loss of bS21-2), reporter gene activity is unaffected by the presence or absence of bS21-2 (**Figure 2B**, *tul4*). However, for genes regulated by bS21-2, there are significant changes in reporter activity in the presence or absence of bS21-2 (**Figure 2B**, *pdpA*). Thus, **specific 5' UTRs are sufficient to permit regulation by a bS21 homolog** (**Figure 2B**, *pdpA*). This regulation is lost when specific sequences elements in the 5' UTR are modified (**Figure 2B**, *pdpA* mut2 vs *pdpA* mut1). While this suggests that the sequence of the 5' UTR is key to regulation, many aspects of this regulatory mechanism remain to be determined, including if this regulation is direct or indirect, and what sequences are sufficient to lead to regulation.

bS21 content leads to differential antibiotic sensitivity in *F. tularensis*

Considering the impact of bS21-2 on gene expression, we hypothesized that incorporation of bS21-2 alters the structure or conformation of the ribosome, modifying ribosome activity and leading to changes in translation initiation. To probe for changes in ribosome structure among cells with different bS21 content, we used a set of translation inhibitors that function by binding to specific sites in the 30S subunit. To examine ribosomes

Table 1. Inhibitory zone diameter (mm) of select antibiotics

	WT (all bS21 homologs)	Tn7::bS21-1 (only bS21-1)	Tn7::bS21-2 (only bS21-2)	Tn7::bS21-3 (only bS21-3)
<i>kasugamycin</i>	33.5 +/- 1.4	30.5 +/- 0.4*	34.3 +/- 2.1	39.7 +/- 0.8*
<i>kanamycin</i>	53.5 +/- 0.6	55.3 +/- 0.2	53.6 +/- 0.4	53.2 +/- 0.5
<i>tetracycline</i>	46.0 +/- 0.9	45.8 +/- 1.4	49.0 +/- 1.9	47.2 +/- 0.3
<i>streptomycin</i>	55.1 +/- 3.4	54.9 +/- 0.7	56.1 +/- 0.1	58.0 +/- 1.8
<i>ciprofloxacin</i>	56.5 +/- 1.3	56.0 +/- 1.2	55.1 +/- 0.1	56.0 +/- 0.1

+/- values represent standard deviation; results are representative of at least two replicate experiments performed in biological triplicate
*Students t-test p < 0.05 compared to WT

homogenous for a given bS21 homolog, we created a set of isogenic strains with single bS21-coding genes in the same genomic context. Specifically, these cells lack all three native genes encoding bS21 and, at a neutral location (the Tn7 attachment site), encode a single bS21 homolog (**Table 1**). We tested the sensitivity of these cells to various antibiotics to determine how distinct bS21 homologs influences antibiotic efficacy.

Using disc diffusion assays, we found no differences in antibiotic susceptibility among the isogenic strains for the 30S ribosome inhibitors kanamycin, tetracycline, streptomycin, or the DNA gyrase inhibitor ciprofloxacin. However, we found that cells containing only bS21-3 (Tn7::rpsU3) are significantly more susceptible to the translation initiation inhibitor kasugamycin, while cells containing only bS21-1 are less susceptible (Tn7::rpsU1; **Table 1**). Our findings suggest that incorporation of distinct bS21 homologs into the ribosome leads to structural changes in or around the kasugamycin binding site. **This provides a proof of principle that we may be able to target specific ribosome populations to develop ribosome-specific inhibitors.** If specific populations of ribosomes are necessary for virulence, such as those ribosomes incorporating bS21-2 in *F. tularensis*, compounds that inhibit these specific ribosomes could be effective virulence inhibitors.

III. Overview of the future research program

We propose to answer fundamental questions related to ribosome biology and gene expression.

Our recent progress demonstrates our expertise in a variety of approaches, including multiple “omics” analyses, quantitative protein analysis, ribosome purification techniques, and bacterial genetics. Give this expertise, we propose to extend our current studies to examine several broad questions. In particular, we are interested in (A) How does ribosome heterogeneity impact gene expression? (B) Why is a ribosomal protein non-essential for translation essential for bacterial survival? and (C) How does an r-protein impact cell envelope-targeting antibiotic sensitivity?

During the next five years, we will answer these questions by focusing on a single ribosomal protein, bS21, and its function in different bacterial organisms (**Figure 1**). This work will examine species that may take advantage of fundamental features of translation initiation to use bS21 in distinct ways. The first is the human

pathogen *F. tularensis*, which encodes multiple distinct bS21 homologs. Given these multiple homologs, we will explore the possibility that use of distinct bS21 homologs in translation could provide a mechanism for post-transcriptional regulation. The second is the model organism and potential pathogen *E. coli*, which encodes a single, apparently essential, bS21 protein. This raises the possibility that bS21 in pathogens like *E. coli* could be targeted by antimicrobials. The third organism is the pathobiont *S. aureus*, which encodes a single non-essential bS21 important for sensitivity to certain antimicrobials via an unknown mechanism. Studying the role and function of the same ribosomal protein in these three organisms will lead to novel and key insights into fundamental aspects of translation and how ribosome heterogeneity impacts gene expression, both of which we may be able to leverage in future antibiotic development.

A. The role of multiple bS21 homologs

Scientific Challenge: While most organisms have one or no bS21-encoding gene, a subset of organisms, including human pathogens like *F. tularensis*, the plant pathogen *Agrobacterium tumefaciens*, symbiotic bacteria (e.g. *Sinorhizobium meliloti*), and free-living cells (e.g. *Shewanella oneidensis*), encode multiple bS21 homologs. How incorporation of these homologs into the ribosome impacts gene expression is unknown.

Overall Strategy: We will leverage the Ramsey laboratory's expertise in *F. tularensis* to: (1) identify which transcripts are translated by ribosomes containing particular bS21 homologs using ribosome profiling and (2) determine how bS21 exerts its effects on gene expression at the molecular level using reporter fusion assays and structural studies.

Rationale: bS21 has been implicated in gene regulation in multiple organisms and we have recently demonstrated that bS21 governs expression of important virulence genes in *F. tularensis*. Given our recent progress and expertise in *F. tularensis* biology and genetic manipulation, *F. tularensis* is an ideal model organism to investigate how multiple bS21 homologs impact translation and gene expression at the molecular level.

Experimental Plans:

1. *Using ribosome profiling to determine transcripts translated by ribosomes with particular bS21 homologs.* We will perform ribosome profiling on all the ribosomes in the cell and on ribosomes that only incorporate one of the three bS21 homologs. In order to accomplish this goal with appropriate rigor and minimally perturb ribosome abundance, we will immunoprecipitate each class of ribosomes from the same cells using affinity-tagged versions of bS21 homologs encoded by their native genomic loci. This will ensure that all cells have biologically relevant amounts of total ribosomes and still provide homolog-specific information about translation. We will take advantage of protocols developed specifically for bacterial ribosome profiling^{44,45}. These analyses will be paired with RNA-Seq, to assess relative transcript abundance. Because two bS21 homologs are less abundant in standard *in vitro* conditions, we will identify factors that lead to their increased abundance and perform ribosome profiling on cells grown accordingly. Specifically, we will create fusions between reporter genes (e.g., *gfp*, *lacZ*), and the promoters for each bS21 homolog. We will then test and identify conditions that lead to increased reporter activity, e.g., altered media composition, temperature, and/or mutation by transposon mutagenesis.

2. *Determining the molecular mechanism by which bS21 exerts its effects.* bS21 functions in translation initiation and is located in an ideal location in the 30S subunit to interact with the 5' UTR of translating mRNAs, adjacent to the mRNA exit channel²³. We hypothesize that interactions between certain 5' UTR sequences and bS21 influence translation initiation, a hypothesis supported by recent progress (**Figure 2B**)¹⁸. Incorporating bioinformatics analysis of the data generated in section 1, we will continue to use similar reporter constructs to identify and confirm the sequence elements that are necessary and sufficient for regulation by each bS21 homolog in *F. tularensis*. To address if this regulation is the result of direct or indirect interactions between bS21 and the 5' UTR, we will take advantage of a commercially available *in vitro* translation kit (NEB PURExpress Δ Ribosome kit). This will allow us to examine the impact of purified *F. tularensis* ribosomes with varied bS21 content on translation of specific 5' UTR-reporter transcripts. Finally, to examine how incorporation of distinct bS21 homologs impacts ribosome conformation, we will use chemical footprinting to determine the precise location of different *F. tularensis* bS21 homologs on the ribosome. bS21 binds to the 16S rRNA; exposure of ribosomes to hydroxyl radicals will lead to cleavage at solvent-accessible nucleotides. By comparing the modifications among ribosomes with different bS21 content, we will identify which rRNA nucleotides, if any, have changed conformation (become more or less solvent-accessible) due to changes in bS21. Similarly, we can perform chemical footprinting on ribosomes forming initiation complexes with mRNA to determine how bS21 content impacts the conformation of the mRNA.

Expected Results: These studies would be the first to identify how changes in a single r-protein impact translation across the genome and to determine how bS21 exerts its effects on gene expression at the molecular level. Our ribosome profiling results will identify which genes are directly regulated by particular bS21 homologs. This will provide a robust dataset to computationally analyze the regulated genes and bioinformatically predict

what sequence elements lead to regulation by particular bS21 homologs. We will then test these predictions experimentally using reporter assays, *in vitro* and *in vivo*. The structural studies of the ribosome-bS21 homolog interactions will provide insight into the molecular basis for the effects of bS21 on gene expression.

Potential problems and alternative strategies: When immunoprecipitating each bS21 homolog for ribosome profiling, there is likely to be different efficiencies of pull-down for each epitope tag. To ensure rigor and reproducibility, we will repeat these experiments after switching epitope tags, such that we IP each homolog with each tag. Ribosome profiling is technically challenging but the Ramsey lab has already demonstrated expertise in ribosome isolation, protein purification, and next-generation sequencing techniques^{18,46,47}. In the event of additional challenges, Dr. Ramsey will consult with Dr. Allen Buskirk, an expert in ribosome profiling. For advice regarding chemical probing, Dr. Ramsey will consult with Dr. Steven Gregory, an expert in ribosome biology.

Significance and long-term goals: The detailed studies proposed here are expected to reveal how ribosome heterogeneity within a single organism leads to gene regulation at the molecular level. Demonstrating that ribosomes can function as programmable regulatory machines would represent a significant innovation and a major paradigm shift in our understanding of bacterial gene expression. We expect our future work extending from this project would include how bS21 homologs are regulated in *F. tularensis*, additional structural studies on *F. tularensis* ribosomes (including using cryoEM to solve the structure ribosomes with each bS21 homolog, with and without antibiotics such as kasugamycin), and studies of multiple bS21 homologs in other organisms.

B. The requirement for bS21 in cells

Scientific Challenge: While the single bS21-encoding gene in *E. coli* is apparently essential, bS21 is dispensable for translation *in vitro*. Why bS21 is essential for viability, but not translation, is unknown.

Overall Strategy: We will (1) confirm the essentiality of bS21 in *E. coli* and (2) compare genome-wide translation by ribosomes with and without bS21 using ribosome profiling.

Rationale: The gene encoding bS21 has been lost multiple times across the bacterial phylogeny, strongly suggesting bS21 is non-essential^{7,26,27}. Furthermore, *E. coli* ribosomes do not need bS21 for translation; *in vitro*, ribosomes lacking bS21 are competent for translation^{21,48}. Yet attempts to generate *E. coli* without the single gene encoding bS21 have not been successful, strongly indicating it is essential for robust cell growth^{49–51}. We have generated *F. tularensis* cells lacking the syntenic bS21 homolog, indicating that the inability to create a deletion mutant is not due to polar effects¹⁸. Potential explanations for this discrepancy include that bS21 is necessary to initiate translation of essential genes, or that ribosomes without bS21 cannot initiate translation *in vivo*. By performing ribosome profiling selectively on ribosomes with or without bS21, we should be able to distinguish between these possibilities.

Experimental Plan:

1. *Confirming the essentiality of bS21 in E. coli.* While multiple attempts to delete the gene encoding bS21 (*rpsU*) have been unsuccessful, we will formally test the essentiality of this gene using a well-described ClpXP-based depletion approach⁵². In particular, we will fuse a degradation tag (DAS4) to the C-terminus of bS21, in a strain with inducible SspB. When production of the SspB chaperone is induced, it will lead to degradation of the tagged bS21. This will allow us to assess cell survival upon depletion of bS21.

2. *Using ribosome profiling to compare genome-wide translation of ribosomes with and without bS21.* Similarly to our study of multiple bS21 homologs in section A, we will assess translation using RNA-Seq and ribosome profiling. In particular, we will compare ribosomes immunoprecipitated with epitope-tagged bS21 to ribosomes that were not immunoprecipitated.

Expected Results: In our initial experiments, we expect that loss of bS21 will result in non-viable cells. By performing ribosome profiling on ribosomes with and without bS21, we will be investigating both the role of an essential bS21 homolog as well as ribosome heterogeneity in *E. coli*. We expect that our analyses will either identify essential genes preferentially translated by ribosomes containing bS21 or a relative defect in translation by ribosomes without bS21.

Potential problems and alternative strategies: It is possible that cells lacking bS21 are viable but grow poorly. The question of why bS21 is important for normal rates of cellular growth, but not for translation, would still lead to insight regarding the role of bS21 in translation. Another potential issue is that immunoprecipitating ribosomes with bS21 may not remove all the bS21-containing ribosomes from the lysate. We will optimize the immunoprecipitation conditions such that the remaining lysate is significantly enriched for ribosomes lacking bS21. If there are not sufficient ribosomes lacking bS21, we will use our depletion strategy to reduce the amount of bS21 in cells prior to performing ribosome profiling. There are other possible reasons bS21 is essential not described above (e.g., bS21 has another function). We expect our analyses will still reveal the molecular basis for the importance of bS21. The Ramsey laboratory has significant expertise in bacterial genetics but we will consult with Dr. Steven Gregory, an expert in *E. coli* genetics, should we encounter any issues.

Significance and long-term goals: These studies are expected to determine why a ribosomal protein non-essential for translation *in vitro* is critical for cell growth. These results may change our understanding of what constitutes the essential translation machinery in cells. If bS21 is essential for survival of *E. coli*, it raises the possibility that bS21 could be a novel drug target and we may be able to identify a species-specific drug by targeting *E. coli* bS21. Future areas of investigation may include identifying the mechanism for preferential translation of certain mRNAs (similar to section A), identifying differences between *in vitro* and *in vivo* translation initiation, and creating assays to screen for novel antimicrobials targeting ribosomes with bS21.

C. The function of a non-essential bS21 in antibiotic sensitivity

Scientific Challenge: Clinical isolates of *S. aureus* with increased vancomycin and daptomycin resistance have been identified with loss-of-function mutations in the gene encoding bS21. How loss of a ribosomal protein impacts the efficacy of cell envelope-targeting antibiotics is unknown.

Overall Strategy: We will (1) confirm that loss of bS21 in *S. aureus* leads to increased vancomycin and daptomycin resistance, and (2) determine how bS21 influences sensitivity to cell envelope-targeting antibiotics.

Rationale: In *S. aureus*, bS21 is not essential and its loss or mutation results in resistance to cell-wall targeting antibiotics through an unknown mechanism. It is possible that loss of bS21 leads to changes in gene expression that result in increased resistance through known pathways, such as modification of the cell envelope.

Experimental Plan:

1. Confirm the resistance of cells lacking bS21 to cell envelope-targeting antibiotics. Multiple studies indicate that loss of functional bS21 leads to increased vancomycin and daptomycin resistance^{32–37}. We will validate this observation by creating a clean deletion of the gene encoding bS21 and assessing vancomycin and daptomycin sensitivity compared to wild-type cells.

2. Determine how loss of bS21 impacts sensitivity to cell envelope-targeting antibiotics. Modified cell envelopes, and particularly increases in cell wall thickness, are a well-described mechanism leading to increased vancomycin resistance^{53,54}. To determine if cells lacking bS21 are more resistant to cell envelope-targeting antibiotics due to increased cell wall thickness, we will use electron microscopy to compare cell wall thickness in cells with and without bS21. We will also take unbiased whole-genome approaches to determine what changes in gene expression lead to altered antibiotic resistance (and potentially cell wall modifications). In particular, similarly to our study of *E. coli* bS21 in section B, we will immunoprecipitate ribosomes with epitope-tagged bS21 and use ribosome profiling and RNA-Seq to assess transcript abundance and translation. We will compare these results with the ribosomes that were not immunoprecipitated.

Expected Results: In our initial experiments, we expect that loss of bS21 will result in increased resistance to vancomycin and daptomycin. We anticipate that this may be due to increased cell wall thickness of cells lacking bS21. Regardless of cell wall thickness, we will investigate the role of a non-essential bS21 homolog as well as ribosome heterogeneity on gene expression in *S. aureus* by performing ribosome profiling on ribosomes with and without bS21. We expect that our analyses will either identify differences in translation for mRNAs that lead to changes in cell wall thickness or other known modulators of drug resistance⁵⁵.

Potential problems and alternative strategies: While mutations in bS21 have been implicated in increased vancomycin and daptomycin resistance in multiple studies, it is possible that we will not be able to replicate these phenotypes with a clean deletion. In this case, we will create the mutations that were identified in the clinical isolates. The Ramsey laboratory has significant expertise in bacterial genetics, but if issues related to *S. aureus* genetics arise, we will consult with Dr. Thomas Bernhardt, an expert in bacterial cell wall biology with significant expertise in *S. aureus* genetics.

Significance and long-term goals: Antibiotic-resistant *S. aureus* are a significant issue in healthcare. Understanding the mechanisms that lead to this resistance will allow us to develop improved novel antimicrobials or drugs that restore sensitivity to current antibiotics. In determining how a ribosomal protein can influence the efficacy of cell envelope-targeting antibiotics, we expect to better understand the regulatory mechanisms that lead to resistance and identify promising drug targets. In addition, we expect the ribosome profiling experiments will provide innovative information to understand how bS21 post-transcriptionally regulates gene expression.

Summary

My proposed research plan takes advantage of a single poorly-understood ribosomal protein to address both specific and open-ended questions. These studies will provide innovative information about how the ribosome functions, how ribosome heterogeneity impacts gene expression, and potentially identify novel targets for species-specific or ribosome-specific antibiotics. In addition to potential future areas of investigation outlined above, these studies may lead us into projects continuing to study bS21 in other organisms (i.e., bacteriophage) or investigate the impacts of other sources of ribosome heterogeneity. Together, our work will have a significant impact the fields of ribosome biology, gene expression, and antibiotic development.

REFERENCES CITED

1. Murray CJ, Ikuta KS, Sharara F, Swetschinski L, Aguilar GR, Gray A, Han C, Bisignano C, Rao P, et al. *Global burden of bacterial antimicrobial resistance in 2019: a systematic analysis*. Lancet. 2022;399(10325):629–655. PMID: PMC8841637
2. Hotinger JA, Morris ST, May AE. *The Case against Antibiotics and for Anti-Virulence Therapeutics*. Microorganisms. 2021;9(10):2049. PMID: PMC8537500
3. Lin J, Zhou D, Steitz TA, Polikanov YS, Gagnon MG. *Ribosome-Targeting Antibiotics: Modes of Action, Mechanisms of Resistance, and Implications for Drug Design*. Annu Rev Biochem. 2018;87(1):1–28. PMID: 29570352
4. Ramakrishnan V. *The Ribosome Emerges from a Black Box*. Cell. 2014;159(5):979–984. PMID: 25416939
5. Jobe A, Liu Z, Gutierrez-Vargas C, Frank J. *New Insights into Ribosome Structure and Function*. Cold Spring Harb. Perspect. 2019;11(1):a032615. PMID: PMC6314068
6. Lilleorg S, Reier K, Pulk A, Liiv A, Tammsalu T, Peil L, Cate JHD, Remme J. *Bacterial ribosome heterogeneity: Changes in ribosomal protein composition during transition into stationary growth phase*. Biochimie. 2019;156:169–180. PMID: 30359641
7. Yutin N, Puigbo P, Koonin EV, Wolf YI. *Phylogenomics of prokaryotic ribosomal proteins*. PLoS One. 2012;7(5):e36972. PMID: PMC3353972
8. Barna M, Karbstein K, Tollervey D, Ruggero D, Brar G, Greer EL, Dinman JD. *The promises and pitfalls of specialized ribosomes*. Mol Cell. 2022;82(12):2179–2184. PMID: 35714581
9. Chen YX, Xu Z, Ge X, Hong JY, Sanyal S, Lu ZJ, Javid B. *Selective translation by alternative bacterial ribosomes*. Proc National Acad Sci. 2020;117(32):19487–19496. PMID: PMC7431078
10. Gabriel SE, Helmann JD. *Contributions of Zur-controlled ribosomal proteins to growth under zinc starvation conditions*. J Bacteriol. 2009;191(19):6116–22. PMID: PMC2747910
11. Dow A, Pristic S. *Alternative ribosomal proteins are required for growth and morphogenesis of Mycobacterium smegmatis under zinc limiting conditions*. PLoS One. 2018;13(4):e0196300. PMID: PMC5912738
12. Dow A, Burger A, Marcantonio E, Pristic S. *Multi-Omics Profiling Specifies Involvement of Alternative Ribosomal Proteins in Response to Zinc Limitation in Mycobacterium smegmatis*. Front Microbiol. 2022;13:811774. PMID: PMC8866557
13. Panina EM, Mironov AA, Gelfand MS. *Comparative genomics of bacterial zinc regulons: Enhanced ion transport, pathogenesis, and rearrangement of ribosomal proteins*. Proc National Acad Sci. 2003;100(17):9912–9917. PMID: PMC187884
14. Natori Y, Nanamiya H, Akanuma G, Kosono S, Kudo T, Ochi K, Kawamura F. *A fail-safe system for the ribosome under zinc-limiting conditions in Bacillus subtilis*. Mol Microbiol. 2007;63(1):294–307. PMID: 17163968
15. Nanamiya H, Kawamura F. *Towards an Elucidation of the Roles of the Ribosome during Different Growth Phases in Bacillus subtilis*. Biosci Biotechnology Biochem. 2010;74(3):451–461. PMID: 20208344
16. Owen GA, Pascoe B, Kallifidas D, Paget MSB. *Zinc-Responsive Regulation of Alternative Ribosomal Protein Genes in Streptomyces coelicolor Involves Zur and sigmaR*. J Bacteriol. 2007;189(11):4078–4086. PMID: PMC1913420
17. Pristic S, Hwang H, Dow A, Barnaby O, Pan TS, Lonzanida JA, Chazin WJ, Steen H, Husson RN. *Zinc regulates a switch between primary and alternative S18 ribosomal proteins in Mycobacterium tuberculosis*. Mol Microbiol. 2015;97(2):263–80. PMID: PMC4548965
18. Trautmann HS, Ramsey KM. *A Ribosomal Protein Homolog Governs Gene Expression and Virulence in a Bacterial Pathogen*. J Bacteriol. 2022;e00268-22. PMID: 36121290
19. Ban N, Beckmann R, Cate JH, Dinman JD, Dragon F, Ellis SR, Lafontaine DL, Lindahl L, Liljas A, Lipton JM, McAlear MA, Moore PB, Noller HF, Ortega J, Panse VG, Ramakrishnan V, Spahn CM, Steitz TA, Tchorzewski M, Tollervey D, Warren AJ, Williamson JR, Wilson D, Yonath A, Yusupov M. *A new system for naming ribosomal proteins*. Curr Opin Struct Biol. 2014;24:165–169. PMID: PMC4358319

20. Chang C, Craven GR. *Identification of several proteins involved in the messenger RNA binding site of the 30 S ribosome by inactivation with 2-methoxy-5-nitro tropone*. J Mol Biol. 1977;117(2):401–18. PMID: 604509
21. Van Duin J, Wijnands R. *The function of ribosomal protein S21 in protein synthesis*. Eur J Biochem. 1981;118(3):615–9. PMID: 7028483
22. Berk V, Zhang W, Pai RD, Cate JHD, Cate JHD. *Structural basis for mRNA and tRNA positioning on the ribosome*. Proc National Acad Sci . 2006;103(43):15830–15834. PMCID: PMC1635088
23. Watson ZL, Ward FR, Méheust R, Ad O, Schepartz A, Banfield JF, Cate JH. *Structure of the bacterial ribosome at 2 Å resolution*. Elife. 2020;9:e60482. PMCID: PMC7550191
24. Kaledhonkar S, Fu Z, Caban K, Li W, Chen B, Sun M, Gonzalez RL, Frank J. *Late steps in bacterial translation initiation visualized using time-resolved cryo-EM*. Nature. 2019;570(7761):400–404. PMID: 31108498
25. Held WA, Ballou B, Mizushima S, Nomura M. *Assembly mapping of 30 S ribosomal proteins from Escherichia coli. Further studies*. J Biological Chem. 1974;249(10):3103–11. PMID: 4598121
26. Mears JA, Cannone JJ, Stagg SM, Gutell RR, Agrawal RK, Harvey SC. *Modeling a Minimal Ribosome Based on Comparative Sequence Analysis*. J Mol Biol. 2002;321(2):215–234. PMID: 12144780
27. Galperin MY, Wolf YI, Garushyants SK, Alvarez RV, Koonin EV. *Nonessential Ribosomal Proteins in Bacteria and Archaea Identified Using Clusters of Orthologous Genes*. J Bacteriol. 2021;203(11). PMID: 33753464
28. Robertson WR, Dowsett SJ, Hardy SJS. *Exchange of ribosomal proteins among the ribosomes of Escherichia coli*. Mol. Gen. Genet. 1977 Nov 29;157(2):205–214. PMID: 340925
29. Pulk A, Liiv A, Peil L, Maiväli Ü, Nierhaus K, Remme J. *Ribosome reactivation by replacement of damaged proteins*. Mol Microbiol. 2010;75(4):801–814. PMID: 19968789
30. Akanuma G, Nanamiya H, Natori Y, Yano K, Suzuki S, Omata S, Ishizuka M, Sekine Y, Kawamura F. *Inactivation of Ribosomal Protein Genes in Bacillus subtilis Reveals Importance of Each Ribosomal Protein for Cell Proliferation and Cell Differentiation*. J Bacteriol. 2012;194(22):6282–6291. PMCID: PMC3486396
31. Takada H, Morita M, Shiwa Y, Sugimoto R, Suzuki S, Kawamura F, Yoshikawa H. *Cell motility and biofilm formation in Bacillus subtilis are affected by the ribosomal proteins, S11 and S21*. Biosci. Biotechnol. Biochem. 2014;78(5):898–907. PMID: 25035996
32. Blake KL, O'Neill AJ. *Transposon library screening for identification of genetic loci participating in intrinsic susceptibility and acquired resistance to antistaphylococcal agents*. J Antimicrob Chemoth. 2013;68(1):12–16. PMID: 23045225
33. Cameron DR, Ward DV, Kostoulas X, Howden BP, C. JrM R, Eliopoulos GM, Peleg AY. *Serine/threonine phosphatase Stp1 contributes to reduced susceptibility to vancomycin and virulence in Staphylococcus aureus*. J Infect Dis. 2012;205(11):1677–87. PMCID: PMC3415852
34. Hiramatsu K, Kayayama Y, Matsuo M, Aiba Y, Saito M, Hishinuma T, Iwamoto A. *Vancomycin-intermediate resistance in Staphylococcus aureus*. J Glob Antimicrob Re . 2014;2(4):213–224. PMID: 27873679
35. Matsuo M, Cui L, Kim J, Hiramatsu K. *Comprehensive identification of mutations responsible for heterogeneous vancomycin-intermediate Staphylococcus aureus (hVISA)-to-VISA conversion in laboratory-generated VISA strains derived from hVISA clinical strain Mu3*. Antimicrob Agents Ch. 2013;57(12):5843–53. PMCID: PMC3837870
36. Basco MDS, Kothari A, McKinzie PB, Revollo JR, Agnihothram S, Azevedo MP, Saccente M, Hart ME. *Reduced vancomycin susceptibility and increased macrophage survival in Staphylococcus aureus strains sequentially isolated from a bacteraemic patient during a short course of antibiotic therapy*. J Med Microbiol. 2019;68(6):848–859. PMID: 31136294
37. Friedman L, Alder JD, Silverman JA. *Genetic Changes That Correlate with Reduced Susceptibility to Daptomycin in Staphylococcus aureus*. Antimicrob Agents Ch. 2006;50(6):2137–2145. PMCID: PMC1479123
38. Gutierrez MG, Yoder-Himes DR, Warawa JM. *Comprehensive identification of virulence factors required for respiratory melioidosis using Tn-seq mutagenesis*. Front. Cell. Infect. Microbiol. 2015;5(164):78. PMCID: PMC4631991

39. Metselaar KI, Besten HMW den, Boekhorst J, Hijum SAFT van, Zwietering MH, Abee T. *Diversity of acid stress resistant variants of Listeria monocytogenes and the potential role of ribosomal protein S21 encoded by rpsU*. Front Microbiol. 2015;6(75):422. PMID: PMC4424878
40. Jha V, Roy B, Jahagirdar D, McNutt ZA, Shatoff EA, Boleratz BL, Watkins DE, Bundschuh R, Basu K, Ortega J, Fredrick K. *Structural basis of sequestration of the anti-Shine-Dalgarno sequence in the Bacteroidetes ribosome*. Nucleic Acids Res. 2020;49(1):547–567. PMID: 33330920
41. Mizuno CM, Guyomar C, Roux S, Lavigne R, Rodriguez-Valera F, Sullivan MB, Gillet R, Forterre P, Krupovic M. *Numerous cultivated and uncultivated viruses encode ribosomal proteins*. Nat Commun. 2019;10(1):752. PMID: PMC6375957
42. Al-Shayeb B, Sachdeva R, Chen LX, Ward F, Munk P, Devoto A, Castelle CJ, Olm MR, Bouma-Gregson K, Amano Y, He C, Méheust R, Brooks B, Thomas A, Lavy A, Matheus-Carnevali P, Sun C, Goltsman DSA, Borton MA, Sharrar A, Jaffe AL, Nelson TC, Kantor R, Keren R, Lane KR, Farag IF, Lei S, Finstad K, Amundson R, Anantharaman K, Zhou J, Probst AJ, Power ME, Tringe SG, Li WJ, Wrighton K, Harrison S, Morowitz M, Relman DA, Doudna JA, Lehours AC, Warren L, Cate JHD, Santini JM, Banfield JF. *Clades of huge phages from across Earth's ecosystems*. Nature. 2020;578(7795):425–431. PMID: PMC7162821
43. Chen LX, Jaffe AL, Borges AL, Penev PI, Nelson TC, Warren LA, Banfield JF. *Phage-encoded ribosomal protein S21 expression is linked to late-stage phage replication*. ISME Commun. 2022;2(1):31.
44. Mohammad F, Green R, Buskirk AR. *A systematically-revised ribosome profiling method for bacteria reveals pauses at single-codon resolution*. Elife. 2019;8:e42591. PMID: PMC6377232
45. Hwang JY, Buskirk AR. *A ribosome profiling study of mRNA cleavage by the endonuclease RelE*. Nucleic Acids Res. 2017 Jan 9;45(1):327–336.
46. Travis BA, Ramsey KM, Prezioso SM, Tallo T, Wandzilak JM, Hsu A, Borgnia M, Bartesaghi A, Dove SL, Brennan RG, Schumacher MA. *Structural Basis for Virulence Activation of Francisella tularensis*. Mol Cell. 2021;81(1):139–152.e10. PMID: PMC7959165
47. Ramsey KM, Ledvina HE, Tresko TM, Wandzilak JM, Tower CA, Tallo T, Schramm CE, Peterson SB, Skerrett SJ, Mougous JD, Dove SL. *Tn-Seq reveals hidden complexity in the utilization of host-derived glutathione in Francisella tularensis*. Plos Pathog. 2020;16(6):e1008566. PMID: PMC7340319
48. Shimojo M, Amikura K, Masuda K, Kanamori T, Ueda T, Shimizu Y. *In vitro reconstitution of functional small ribosomal subunit assembly for comprehensive analysis of ribosomal elements in E. coli*. Commun Biology. 2020;3(1):142. PMID: PMC7096426
49. Bubunenko M, Baker T, Court DL. *Essentiality of ribosomal and transcription antitermination proteins analyzed by systematic gene replacement in Escherichia coli*. J. Bacteriol. 2007 Apr;189(7):2844–2853.
50. Yamamoto N, Nakahigashi K, Nakamichi T, Yoshino M, Takai Y, Touda Y, Furubayashi A, Kinjyo S, Dose H, Hasegawa M, Datsenko KA, Nakayashiki T, Tomita M, Wanner BL, Mori H. *Update on the Keio collection of Escherichia coli single-gene deletion mutants*. Mol Syst Biol. 2009;5(1):335. PMID: PMC2824493
51. Goodall ECA, Robinson A, Johnston IG, Jabbari S, Turner KA, Cunningham AF, Lund PA, Cole JA, Henderson IR. *The Essential Genome of Escherichia coli K-12*. MBio. 2018;9(1):e02096-17. PMID: PMC5821084
52. McGinness KE, Baker TA, Sauer RT. *Engineering controllable protein degradation*. Mol. Cell. 2006 Jun 9;22(5):701–707.
53. McGuinness WA, Malachowa N, DeLeo FR. *Vancomycin Resistance in Staphylococcus aureus*. Yale J Biology Medicine. 2017;90(2):269–281. PMID: PMC5482303
54. Tran TT, Munita JM, Arias CA. *Mechanisms of drug resistance: daptomycin resistance*. Ann Ny Acad Sci. 2015;1354(1):32–53. PMID: PMC4966536
55. Mlynarczyk-Bonikowska B, Kowalewski C, Krolak-Ulinska A, Marusza W. *Molecular Mechanisms of Drug Resistance in Staphylococcus aureus*. Int J Mol Sci. 2022;23(15):8088. PMID: PMC9332259