Slide 1

Hi everyone. I'm Hannah, and I will be presenting on my research into a small ribosomal protein, bs21 and its role regulating genes

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First I'll start with some background info about our current understanding of gene regulation in bacteria. I'll introduce you to the idea that ribosomes may be heterogeneous in composition and function, and then focus in on a specific source of that heterogeneity, a ribosomal protein called bS21.

Then we will get into my specific questions about how bS21 may be involved in regulating gene expression in two bacteria: F. tularensis and S. aureus, and the implications that has on virulence and antibiotic resistance.

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Here we have a cartoon of any bacterial gene and it's promoter, which is transcribed by RNA polymerase, generating the growing mRNA transcript that you see here. A lot is known about how gene expression is regulated by transcription, but post-transcriptionally, there can also be regulation occurring and this is less well-understood.
For instance, sRNAs can bind to transcripts to promote or prevent the ribosome from binding to translate. There can be RNA binding proteins that influence stability, and differences in mRNA recruitment to the ribosome.

Often people think of the ribosome as constitutively and homogenously being active, but we wonder if ribosomes can provide an element of gene regulation? Structurally, we believe it is possible because ribosomes are heterogeneous., and I'll show you the source of that heterogeneity now.

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First, we can have multiple rRNA operons whose products are incorporating into different ribosomes. These can be post-transcriptionally modified through acetylation or methylation. We can also have homologous ribosomal proteins that incorporate into ribosomes, and these can also be modified, post-translationally.

All these different types of ribosomes that can be made has led the field to start investigating whether these have different functions and, rather than ribosomes acting constitutively as was traditionally believed, perhaps these ribosomes can impart some level of control of gene expression. The term for this is specialized ribosomes, referring to ones that are heterogeneous in structure and in function.

Now heterogeneous ribosomes are well-studied in eukaryotes, with significantly less work being done in bacteria. I want to show you a specific example of this that has been described in bacteria.

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In mycobacterium tuberculosis, there are two genes that encode the small ribosomal protein S18 – the primary ribosomal protein can coordinate with zinc and the alternate ribosomal protein cannot. So in high zinc conditions, which may occur when bacteria are living inside a macrophage, zur, a transcription factor, binds to zinc to repress the alternate S18, and the primary S18 coordinates with zinc, leading to a population of ribosomes with mostly the primary S18 present. In other conditions, perhaps stressful extracellular conditions with low zinc presence, it's been shown that this zur-zinc complex is not present so there is expression of the alternate S18, and a more evenly heterogeneous ribosome population.

The functional consequences of this are not known, but it is hypothesized that this was adapted as a way for mycobacterium to better grow in specific niches, such as in or out of host macrophage cells.

Our research is aimed at another ribosomal protein, called bS21.

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bS21, which is encoded by the gene rpsU, is a component of the small ribosomal subunit. Here we have images of the 30S subunit of the E. coli ribosome. On the right we have a view of only the 16s rRNA with bS21 which is colored in red. On the left is a closer view, and you can see bS21 is positioned close to mRNA exit channel and nearby the tRNA in the p-site.

Early research into the functions of ribosomal proteins identified that bS21 is involved in translation initiation, perhaps moderating the interaction between the ribosome and the mRNA molecule. However, bS21 is not found in all bacterial species and is considered non-essential.

One really interesting study shows that bS21 has been encoded by viruses – different homologs of the gene that encodes bS21 has been found on more than 1300 viral genomes. It has been show that at least one of these viral bS21 can be incorporated into the E. coli ribosome, suggesting that perhaps viruses are utilizing bS21 homologs to hijack the host ribosomes to somehow benefit them, maybe altering gene expression.

Despite the fact that bS21 is not found in all species, there have been a number of studies where null-mutants have been made in species with bS21, and these result in interesting phenotypes, including loss of motility, biofilm formation, changes in acid stress resistance, antibiotic-resistance, and loss of virulence. These last two are going

to be the focus of our studies, but given its influence on specific phenotypes, bS21's role in translation may be a regulatory role.

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My overarching question is if, and how, bS21 contributes to control of gene expression in two pathogenic bacteria.

First I'll be looking at F. tularensis, which I'll introduce you to shortly, where we have 3 paralogs of bS21 and we have seen effects of loss of one of these paralogs on abundance of specific proteins, including one called PdpA, so we would like to look into the mechanism by which this regulation occurs.

The second aim will look at staph aureus, where bS21 has been implicated in resistance to antibiotics.

We'll start with aim 1 with some background information on Francisella.

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Francisella is a **gram -, facultative intracellular bacteria.** It is pathogenic and causes a disease called tularemia, which can take a number of forms based on the route of infection. If infection is via the skin like through a mosquito or tick bite, it produces the ulceroglandular form of the disease, or if infection is by breathing in the bacteria, it causes the respiratory form, which can be fatal.

There are a large number of hosts and vectors including ticks, mosquitos, flies, rabbits, and a number of rodents. It can also be water-borne, which makes understanding its epidemiology rather complex.

It is highly infectious with as few as 10 organisms able to cause the disease, and for this reason, along with the fact that it is potentially lethal, it is considered as a potential bioweapon. This image here is colored so that the francisella are blue and are in a yellow macrophage, and you can clearly see that a ton of organisms replicate.

Luckily we don't have to work with the pathogenic form because there is a live vaccine strain that we can work with in the lab. It's non pathogenic to humans but **retains it's pathogenicity** in animal models, so we can continue to use it in **infection assays**.

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Even though francisella has a very small genome, it encodes three copies of the rpsU genes, which encode bS21. This is especially noteworthy because most bacteria have 0 or 1 of this gene.
We originally hypothesized, and have since shown, that these three proteins are produced and incorporated into ribosomes. This is a western blot of purified ribosomes from our wild-type strain as well as strains **ectopically expressing** each of the bS21 proteins, with a VSVG tag. This, along with some mass spec data we have of purified ribosomes, indicates that F. tularensis can incorporate each paralog into ribosomes.

But how do we think this is related to regulation of gene expression? I will now show you a model we have proposed for how bS21 may play a role in gene regulation.

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As I said the bS21 proteins are important in initiation of translation. So perhaps we have **RNA polymerase actively transcribing a gene.** The 30S subunit that contains paralog 1 **may interact with** th**e leader sequence** of this mRNA – but perhaps there **isn't strong affinity**, so translation doesn't initiate.

Then a 30S with a different bS21 comes in. There could be a stronger affinity, so the 30S interacts with the mRNA long enough for the 50S subunit to come in, **translation is initi**ated, and we see the protein being expressed.

This is a simple model, but it shows our hypothesis: that each bS21 paralog possibly has a greater affinity for distinct mRNA species, leading to preferential translation which results in differences in protein abundance.

Now that I've told you why we are interested in this, I'll show you some of our preliminary data.
We started by individually deleting each of the rpsU genes, looking at global protein abundance, and found something interesting when deleting one of the paralogs.

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Here you have a Coomassie stain of whole cell extracts of our wild-type cells and drpsU2, and we don't see large differences, but we did see a difference in a band here. We sent that out for mass spec and found, among the differences was pdpB, and that there **was substantially less** of this protein in drpsU2 when compared with wild-type.

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Now this was really exciting because pdpB is part of the Francisella pathogenicity island, or FPI. The FPI encodes the type vi secretion system in francisella, and is absolutely essential to virulence. I also wanted to point out here, because this will be important later on, that the **FPI is comprised of two distinct operons.** pdpB is on the plus strand operon here, along with pdpA, and a number of other genes, and the second operon is comprised of iglA-D.

We do know a fair amount about how the FPI genes are regulated at the level of transcription, and I don't want to get too into the details here, but I will just introduce you to a protein called pigR which has been shown to be **necessary for transcription of both operons of the FPI**, so it's a useful control we'll be using throughout these next few experiments. There **haven't been any publications** into whether the FPI is controlled at the level of translation.

Once we identified pdpB as potentially being influenced by the presence of rpsU2, we wanted to confirm these results using immunoblotting, and look to see if any of the other FPI proteins are also affected.

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Here we have the results of those western blots. We ran whole cell lysates in duplicate, our wild-type cells are represented here, and we have antibodies for a few proteins from each of the FPI operons. And then LpnA, is our loading control.

We ran a **control** with deltapigR, because as I mentioned **the pigR protein necessary** for transcription of the **both operons** of the FPI, and you can see clearly that without it, these proteins are not present.

When we compare the wild-type cells to drpsU2, we see a significant reduction of pdpB and a similar loss of pdpA. But, we did **not** observe the same level of difference in protein abundance for the igl proteins, from operon 2. I realize these results are a bit variable and we are working to replicate and quantify these differences.

Now this is all very interesting, but we don't know if this affect on pdpB and pdpA is happening at the level of transcription or translation, so we isolated RNA, generated cDNA, and determined abundance of these different transcripts using qRT-PCR.

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Alright so what you see here first are pdpA and B which were most affected by loss of rpsU2. We are using wild-type and delta pigR as our controls, because FPI proteins require PigR for expression at the level of transcription. And what we see here is that there really is **NOT a significant difference** in transcript abundance of these genes between wild-type and the strain lacking rpsU2.

We also looked at transcript abundance of iglA which is one of the genes on the **other FPI operon** and this also appears to be true.

Finally for controls we looked at two **non-FPI genes**, rpoA1 and bfr, and here we see pretty consistent abundance of these transcripts among the three strains.

The large difference that we are seeing in protein abundance of pdpA and pdpB is clearly not due to differences in transcription, and we want to know how this is occurring. This data **opens up the possibility** that regulation is occurring at the level of translation, which we propose in aim 1.

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Now that I've gone through our preliminary data, I want to get back to my first aim, which is to look at the mechanism behind these differences in protein abundance. As a reminder, our hypothesis is that ribosomes containing bS21-2 show a greater affinity for particular genes, in this case we will be focusing on pdpA, and that this leads to preferential translation and increased protein abundance compared to cells with other ribosomes.

The specific experiments that I have proposed aim to answer two sub-questions. First, what component of the mRNA is recognized, directly or indirectly, by bS21 paralogs? We believe, based on the position of bS21 in the ribosome, may be specifically recognizing the 5' UTR or untranslated region of mRNA.

Second, is the ribosome solely responsible for this interaction? Or are there other proteins in the cell necessary for this preferential translation to occur?

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To get at whether the 5'UTR is the component of the mRNA that is recognized, we have proposed to use B-galactosidase assays. We have built strains that have the 5'UTR of pdpA, which appears to be regulated by bS21-2, fused to the lacZ gene, and we have put these constructs both in wild-type cells and those lacking the second paralog at a neutral location.

What we expect to see is ribosomes that contain the second homolog, such as those in wild-type cells, colored in purple, will show a higher affinity for the 5'UTR than other ribosomes. We expect all types of ribosomes to translate lacZ into the B-galactosidase protein, which cleaves a substrate to produce a yellow color, But that we might get more translation in wild-type because of this higher affinity, leading to a greater production of yellow color.

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We have also constructed control strains that fuse lacZ to the UTR of a gene that is unaffected by presence of the second paralog, bfr. We will compare the ratio of the pdpA 5' UTR to the bfr 5'UTR to compare B-galactosidase activity

So that's basically the experimental setup, and I have gotten a little ahead of my proposal and done one of these assays already, which I'll show to you now.

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What we see here is a ratio of the B-galactosidase activity in our pdpA UTR compared to the bfr UTR in both strains. And we see about a 2-fold difference. Which is cool, but it certainly doesn't explain the massive difference we saw in protein abundance of pdpA.

This doesn't mean it's the end of this line of inquiry, though, because there is evidence that pdpA has an alternative transcription start site about 100 base pairs upstream of the one we used to build these constructs, so it may be that bS21-2 shows a greater affinity only for pdpA transcripts with the longer UTR. As next steps I am in the process of generating the strains with the longer pdpA 5'UTR.

Now as a reminder, my second specific question was about whether the ribosome was sufficient to impart the differences in abundance that we see of pdpA.

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To study this, we are going to do an in-vitro assay, with a kit that has all the transcription-translation machinery, and add in a pdpA transcript, as well as ribosomes isolated from double mutant strains. So we have actually purified ribosomes that only have one of each of the paralogs. We can perform the reactions and then immunoblot the samples to see abundance of pdpA. We expect to find that when only bS21-2 is present, there is more pdpA produced than with ribosomes containing only the other two paralogs.

This would tell us that there aren't other proteins involved in the specific interaction between bS21 and mRNA, it is just the ribosome responsible for the changes we see.

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Alright so in summary, we expect to find whether or not bS21 is recognizing the 5' UTR of mRNA molecules, and whether the ribosome is sufficient to explain these differences. This would not only elucidate the role of bS21 in translation, but it would also be the first study in bacteria highlighting that alterations in r-proteins affect gene expression.

This research could lead in some exciting directions. For instance, we may be able to start screening ribosome-inhibitors to see if there is any specificity for ribosomes containing a specific paralog.
We are also working with collaborators to develop a structure to see how this is working at an atomic level. And finally, if F. tularensis perhaps developed these paralogs this as an evolutionary advantage to adapt better to its wide range of environments, what conditions lead to expression of each paralog?

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I also realize that all of our experiments might not show us what we are hoping to find, so if we don't see the changes we expect in the proposed experiments, we have some alternate plans to better study our hypothesis.

First, if we don't find the 5' UTR is sufficient to create the difference we are seeing, it may be the bS21-2 is affecting translation of another protein, such as a protease, that modifies pdpA post-translationally. We can try to find what that protein may be using a proteomics approach with mass spec.

If we find that the ribosome alone isn't sufficient to exhibit the differences we see in pdpA abundance in our in vitro assay, it is very likely that another protein is interacting with bS21 to moderate the recognition of mRNA. We could do an IP using VSV-G beads to pull down other proteins associating with the protein, or we could do transposon

mutagenesis to find what other proteins are necessary for expression of pdpA. **how will we screen for this again? Is it with lacZ somehow**

Hopefully, between all the proposed and backup experiments, we should be able to learn a great deal about the mechanism of gene regulation via bS21 in francisella.

Slide 22 That brings us to my second aim, which is to determine whether bS21 plays a role in antibiotic resistance in Staphylococcus aureus through alterations in cell wall structure

Slide 23 -G+, facultative human pathogen. Normally lives on skin and mucosal membranes of healthy humans.

-Extremely common, estimate that 30% of population carries staph on their skin asymptomatically. When it infects, can be very mild skin boils or life-threatening bacteremia.

-one reason it is so dangerous is because of antibiotic resistance making it difficult or impossible to treat. These are just some of the antibiotics used to treat staph infections, but some staph strains have acquired resistance to every single one. For years methicillin was the main antibiotic being used, but Methicillin-resistant staph aureus, or MRSA, is now extremely common, leading vancomycin and daptomycin to become more widely used. These are the two I'm going to be focusing on for my second aim, because it turns out that clinically, mutations in rpsU are associated with resistance to vancomycin and daptomycin

Slide 24 There have been numerous studies on clinical strains of staph with resistance to vancomycin or daptomycin, that show mutations in rpsU, the ONLY rpsU in s. aureus, with mutations occurring in different parts of the gene as well as in the promoter region. These loss of function mutants implicate loss of rpsU in antibiotic resistance.

These clinical isolates often have numerous mutations so it's hard to single out rpsU as being causative of the antibiotic resistance that is observed.
Fortunately, there has also been a transposon mutagenesis of staph that implicates rpsU ALONE as the mariner transposon inserts at only one place in the genome. This study showed that knocking out rpsU was sufficient to cause both vancomycin and daptomycin resistance.

The question remains, HOW is loss of bS21 causing antibiotic resistance? Research has shown that many cells exhibiting antibiotic resistance have thickened cell walls, an example of which you can see here in vancomycin resistant bacteria. It is believed that

this thickening prevents antibiotics from entering to their target locations. Even in diverse genetic backgrounds, this same mechanism results in antibiotic resistance.

This information has led us to develop a model for what may be happening with bS21 in S. aureus.

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SLOW DOWN
Much like the model I showed you for francisella, here we have **RNA polymerase actively transcribing a gene, specifically a cell wall negative regulator.** The 30S subunit that contains bS21 **may interact with** this mRNA, there is a strong affinity, a protein is made and we have a normal cell wall produced, which is represented by this cartoon. Antibiotics can enter this cell and eventually the cell dies.

You can imagine that there are also 30S subunits that altogether lack bS21. There may be a weaker affinity between these ribosomes and the mRNA, so translation is not initiated the protein is not produced, and this results in a thicker cell wall. In this case, antibiotics are unable to penetrate and enter the cell.

This is our hypothesis – that bS21 regulates expression of proteins involved in cell wall structure, and this in turn has an affect on antibiotic resistance.

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Now that brings us to the specific questions we have for aim 2.
First, we plan to delete rpsU from staph and determine whether we can replicate the results of the transposon mutagenesis study, which showed that without a functional bS21 staph shows resistance to both vancomycin and daptomycin.
Next, we want to assess whether cells lacking bS21 have any alterations in the cell wall thickness. As a reminder, our hypothesis is that cells without bs21 will have thicker cell walls.

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In order to determine whether we see alterations in antibiotic resistance when bS21 is lost, we will setup a very simple population analysis experiment. We will make plates that have different antibiotic concentrations, and then plate cells at different concentrations to determine that minimum concentration of antibiotic that is necessary to prevent growth, or the MIC. Using a range of starting CFU's will allow us to better quantify if there are heterogeneous populations that have increased MICs which are only evident at higher concentrations of cells.

We plan to do this with both antibiotics – daptomycin and vancomycin- with two strains, our wild-type cells and cells lacking rpsU. Based on the results of previous studies, particularly the transposon-mutagenesis study, we expect that the drpsU strain will have a higher MIC for both antibiotics when compared to wild-type.

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For our second specific question, we want to see whether cell wall size is affected by loss of rpsU. To do this experiment, we will again take our wild-type and drpsU cells, which I have as purple cartoons here, and we will fix and stain the cells, represented here on a slide, then we can analyze using transmission electron microscopy. We should be able to measure the cell walls in each of these strains. We expect to find that cells with rpsU, our wild-type strain, will have thinner cell walls than cells lacking rpsU.

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In summary, we hope to reproduce previous research that shows cells lacking functional bS21 have increased resistance to vancomycin and daptomycin, and we hope to look at whether there are also changes in cell wall size.

This would be really exciting because it would draw a connection between the ribosome, and perhaps gene regulation through translation, to antibiotic resistance.

If the results of these experiments support our model, it could lead in some exciting directions. We would definitely want to look at what specific proteins involved in cell envelope structure are being affected by bS21, directly and indirectly, through a proteomics approach.
We would also want to see if this is true in other organisms that are antibiotic resistant, especially vanc-resistant bugs like enterococcus.

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Of course it is possible that these experiments will not support our model. If that's the case, we might want to take a step back from the cell-wall line of inquiry and attempt to figure out what proteins are differentially expressed when rpsU is lost, through a mass spec experiment similar to what we did with Francisella.

We could also take a genetic approaching by using RNA-seq. If bS21 is affecting translation of a transcription factor, we might see significant changes at the level of transcription, indicating an indirect relationship between bS21 and those genes.

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Just to wrap up, the focus of my dissertation research will be on the role that the small ribosomal protein bS21 plays in two pathogenic bacteria. In francisella tularensis, we will look specifically at the second bS21 paralog to try to isolate the mechanism that this r-protein uses to regulate gene expression through B-galactosidase assays and in-vitro translation assays. Here were are focusing on how ribosomal protein heterogeneity influences gene expression and potentially virulence.

In Staphylococcus aureus, we will look at the relationship between bS21 and antibiotic resistance andcell wall size, using population analysis experiments and transmission electron microscopy. This will help us understand how loss of function of r-proteins, and therefore alterations in ribosome composition, influence gene expression and antibiotic resistance.

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