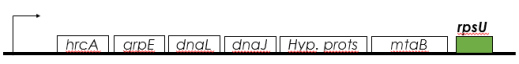
Possible questions for qualifying exam

Francisella:

- Could there be a protein that is less abundant in wild-type cells compared to drpsU2 (f. tul) that is causing upregulation of pdpA and pdpB?
    - Yes, a negative regulator
- How do we know pigR affects transcription of FPI?
    - Transposon mutagenesis study in which iglA was replaced with lacZ and inserted mariner transposon
    - Loss of blue = the gene with that transposon is necessary for iglA expression
- What mutations are in LVS making it non-pathogenic?
    - Made by passing through guinea pigs
    - pilA is mutated (pilus assembly gene)
    - Also an iron uptake/utilization protein
- Size of francisella genome – 1.8 Mbp; S aureus is about 2.8 Mbp; (both have GC contents about 32%); E coli is 4.6 Mbp (GC 50%)
    - Single, circular genomes for all of them
    - Got f tul genome in 2005; got S aureus genome in 2001
- FPI information
    - Two operons in duplicate; 16 genes; pdpD is missing in some strains including LVS
    - Point of the T6SS is to send a phage-like effector out into target cells to hijack host cells
    - iglA + b are part of the sheat, iglC is part of the tube; pdpA is part of spike complex and pdpB is part of membrane
    - tul4/lpna – lipoprotein in membrane – found in all francisella basically
    - Evidence that at least iglE is post-translationally modified – it is a lipoprotein so in the cytoplasmic membrane the signal sequences are cleaved and lipidis added
- -E.Coli bS21 is in same operon **rpoC and primase**, so most similar to #2. Others are more similar to #3, like listeria and bacillus
- - **paralogs** = gene duplication, **orthologs** = from different species. Likely rpsU are paralogs because we built a tree and they all clustered very close together and were only surrounded by other francisella proteins
- -**yqeY** = hypothetical protein, uncharacterized
- DnaG = dna primase; rpoD = sigma factor 70 for RNA polymerase

Staph:

- How many Staph aureus infections? What percentage are MRSA?
    - 120,000 or so per year in the US cause bacteremia, no official number on total numbers of infections
    - Percentage that are MRSA ranges significantly based on region, ranging from 13-75%
- Where is rpsU located in staph aureus?
    - Not clear if it is in an operon all on it's own or part of a very large operon.
    - 

    - What is downstream of rpsU? If there is a polar effect of deleting rpsU?
        - A bunch of hypothetical proteins, then diacylglycerol kinase, cytidine deaminase, GTP-binidng protein era, and another possible protein that could be DNA repair

- Is there an S21 structure or ribosome structure for staph aureus?
    - 70s ribosome was solved in 2016 with cryoEM
    - bS21 was purified but was too mobile so electron density at that location was weak – decided to exclude it.
    - Was modeled in a hibernating ribosome they published a year later
- Are there other sources for ribosome heterogeneity in s aureus?
    - Six or five rRNA copies – some strains of MRSA lose a copy and that seems like it could be related to antibiotic resistance
        - No identified mutations in the overall rRNA but it was hypothesized that loss of a wild-type rRNA leads to greater percentage of possible mutants (less susceptible) in the ribosome population
    - Missing proteins – the solved structure didn't have bS1, uS14, or bL9
    - bL31 has multiple homologs in other gram positives (A vs B type) but S aureus only has B type
    - Obviously, mutations are possible in all of the rRNA and r-proteins
    - Go to RAST.nmpdr.org/seedviewer.cgi and look at ribosome proteins that are duplicated
        - 2 or 3 bL33's – depending on the strain; not identical; 2 coordinate zinc and one doesn't
        - 2 S14's – 1 is zinc-dependent, 1 is not
        - Looking at one strain (N315) which has only 5 16S rRNAs, there is one SNP (c to a t) in one of them.
        - Are any of these implicated in antibiotic resistance? Not that I could find in s. aureus
- Why use HG003 strain for s. aureus?
    - HG003 was generated in 2010 from genomic tools which repaired mutations in a few genes that were used as a model before this one. It's widely considered a good model and is often used by scientists studying staph.
    - IT was originally isolated from a sequence type 8 strain which is the most common sequence type in MRSA in the community but HG003 is not MRSA
    - There's a complete genome sequence for it
- ***Is HG003 pen or strep resistant?
    - Couldn't find specifics, but it seems like most of these strains are pen resistant but may be oxacillin sensitive
- S. aureus strains are VERY diverse
    - Core genome – always found in all strains – about 1400 genes
    - Pangenome – all genes added between strains – about 7000 genes
    - Transposon mutagenesis staph study used SH1000 – has a good amount of variability compared to HG003

Antibiotics:

- What do we already know about vancomycin and daptomycin resistance? **Cong et al 2020
    - Van genes which synthesis d-ala-d-lac and d-ala-d-ser are mutated
        - Two-component system in van genes to regulate
    - Biochemically, van-r genes often have decreased crosslinking of the PG
        - One study also found that van-R had increased production of D-ala-D-ala that were unbound, soaking up vancomycin

    - DAP-R strains are often morphologically different. Suggested genes affected in Dap-R for s. aureus are:
        - dltA-D – D-analylation of cell wall teichoic acids
        - mprF – enzyme involved in lysinylation of phosphatidylglycerol on cell membrane (involved in CM channels)
        - pgsA – gene that synthesizes that same thing - phosphatidylglycerol
        - yycG – two comement system for cell wall synthesis
        - also appears to be repulsion of dap to the cell wall, perhaps by changes in the charge of the cell wall, although this is not well understood (could try comparing cell wall charge of wt to drpsU using titration of electrolyte concentrations and checking pH)
- What methodology was used in mutation studies? Especially basco which was over time?
    - Basco study – patient was diagnosed and treated with vancomycin for 5 days, with blood taken every 2 days. Then switched to daptomycin.
    - Discovered mutation in vraS (sensor in two-component system related to biofilm formation) on day 3 and rpsU on day 5
- VISA vs. VRSA?
    - VISA MIC = 4-8 ug/ml, VRSA = >16
- Is VRSA always MRSA?
    - Most strains are penicillin resistant, so the next line of treatment is usually methicillin. If met resistant, will treat with vancomycin, and that's when you find out vrsa. So generally, yes.
- What mutations were found in the study on cell wall thickness?
    - Overall methodology: generated cells with different thicknesses by incubating in media with different components of cell walls so some could not create de novo peptidoglycan. They didn't actually describe any mutations they found in these, but previous papers have shown mutations or altered expression of pbp2 and pbpD (penicillin binding proteins, cell wall crosslinking), sigB (sigma factor involved in biofilm formation), ddh (involved in making peptidoglycan precursors), tcaA (membrane-bound protein implicated in glycopeptide resistance), and vraSR (two-component system related to biofilm formation, control expression of pili genes) in VISA strains.
- Mechanisms of action of vanc and dapt:
    - Vancomycin was discovered in the 1950s as a cell-wall targeting antimicrobial. We can see hear how a normal non-resistant S. aureus builds peptidoglycan, where you can see new components are added to the growing peptidoglycan via transglycosylation and transpeptidation.
    - Vancomycin hydrogen bonds to the D-ala-D-ala residue on peptidoglycan precursers, thereby preventing transpeptidation and eventually breakdown of the cell wall and lysis.
    - You should not that vancomycin is less effective against gram-positive bacteria because it is unable to penetrate the outer membrane.
    - The mechanism of action of daptomycin is not well understood. There are multiple models that exist in the literature, but they all seem to agree that it targets the cell envelope causing depolarization of the cell membrane. IT is a cyclic lipoprotein that only targets G+ bacteria
    - One of the simple, earlier models believed that this was due to pore formation, though other researchers hypothesized that the breakdown occurred in cell wall synthesis, though the exact step remains unnamed. A more recent study suggests that daptomycin causes fluid lipids to

cluster, thereby decreasing the cell membrane fluidity which in turn can disturb cell wall synthesis and membrane potential

- Which proteins are altered if cell walls are thicker?
    - Can do a whole cell proteomics approach, or specifically target genes we expect to see effected such as those with known association to cell wall size like penicillin binding proteins, ddh which makes PG precursors, etc.
    - May also want to try removing the cell wall and identifying if there are difference in PG structure such as different types of linkages, which may help pinpoint a potential protein faster
- Enterococcus also has only 1 bS21 protein
- Cell wall thickness data –
    - This data is from a study from 2006, in which the researchers used vancomycin-intermediate S. aureus and grew it in two different media, with and without certain cell-wall monomers, generating a thinner cell wall which you see here and one with thickened cell walls, labeled RM.
    - When the cells are grown in normal culture, we see no difference in their growth rates.
    - However, when the cells are grown in the presence of vancomycin, we see that the cells with the thicker cell wall grow better than those with the thinner cell wall.
    - This indicates that there is a correlation between cell wall thickness and antibiotic resistance, specifically vancomycin resistance. However, we are interested in the ribosome. So you might be wondering how does all this talk about antibiotics and staph aureus have to do with our ribosomal protein?

General bS21/ribosome:

- What other proteins does bS21 interact with?
    - 16S rRNA, bS18, uS11
    - I tried to find possible interactions on pymol and most of the residues I thought may be involved in bonding activity with these other ribosomal components are conserved between the e. coli ribosome and the f. tul bS21 ribosomes
- What is the percent identity of rpsU in s aureus compared to francisella genes?
    - Slide at back; 37-46%
- Why do we see an effect on transcription in rpsU-mutated cells?
    - Because the cell is missing functional bS21 so probably tells it to upregulate transcription
- How do we control for alterations in overall number of ribosomes?
    - Changing the number of ribosomes by tagging or knocking out r-proteins can affect translation of SOME mRNA, generating a confounding factor
    - Check overall mRNA levels – could be a sign of ribosome stress. Compare mRNA levels of tagged and untagged cell lines to see if there are effects from tagging your protein on stability. Specifically also look at r-protein transcription.
    - Express each paralog with same non-coding regions – just like we are doing with the Tn7
        - Confirm expression is similar between paralogs by western blotting or mass spec (would need to tag bS21 for western blotting, but could do mass spec)
    - Control for ribosome number by deleting a different ribosomal protein and seeing if the effects occur as well
    - Ensure tagged proteins can be incorporated into ribosomes – we did this already
    - In-vitro assays with tagged proteins should always have un-tagged purified ribosomes
    - Make sure specialized ribosomes are functional – purify and then test in vitro translation assay

- Are there other examples where specialized ribosome inhibitors have been found?
    - I couldn't find any publications about it, although I saw it suggested a number of times in cancer research because it turns out that there are these onco-ribosomes which have mutated L10 proteins
- sRNAs
    - FtrA, B, and C – francisella
    - In s. aureus – about 50 sRNAs are confirmed but some numbers are higher than that
        - RNAIII is an example, it is very important for virulence genes
    - Finding sRNAs you use PAGE – first isolate RNA, deplete rRNAs, elute out small RNAs from 100-200 bps on gel, make cDNA, and send for sequencing; confirm with northern blotting
    - See effects by deleting sRNAs and using microarrays – isolate RNA, make cDNA, label with fluorsence, and then put on a slide with known sequences. If it lights up at that spot, that mRNA is present in your sample
- rRNA variability
    - 7 operons in E. coli with slight variability
    - In step, it was seen that different rrNA molecules are expressed at different stages in development
- Exchange of r-proteins is possible
    - In vitro assay where they chemically inactivated ribosomes then added ribosomal proteins back in, in E. coli, and saw if ribosome activity was restored (used mass spec)
    - bS21 was able to be exchanged
- Mycobacterium experiment
    - Grew cells in different media, and used qPCR to look at expression of each S18 and used Western blotting with antibodies for each S18
    - Myco. Has 4 other r-proteins that seems to have this same zinc-repression phenotype
- bS21 function study
    - Both deleted bS21 and treated with antibodies, then used MS2 mrna which is from a bacteriophage, and basically without bS21 translation didn't occur which pinpoints initiation of translation
        - Were able to show that it was the initiation stage because they pre-incubated RNA and protein before treating with anti-S21 and it was fine
    - Another study modified ribosomes to make inactive then added back in individual r-proteins and were able to bind bacteriophage mRNA – measured mRNA binding by putting with 30S subunits then filtering so only bound mRNAs are kept. The RNAs are radioactively tagged so can be measured with mass spec
- Viral bS21 protein exchange
    - They expressed viral gene on plasmid, then used high salt purification to do ribosome purf and looked for presence of viral bS21 with mass spec
        - Double the ammonium chloride concentration that we use
- Specific phenotypes – which species?
    - In **bacillus subtilis**, this results in defects in motility and biofilm formation (identified by whole genome resquencing of a variant), and in **listeria monocytogenes**, null mutants have increased acid stress resistance and changes in transcription of stress resistance genes (also whole genome resequencing of a variant)
- UTRs

    - 3' UTRs also exist and can even act as sRNAs to affect stability of its own gene
    - Mostly form secondary structures, although evidence that tertiary structures can occur as well such as G-quadriplexes which can affect translation
- What couples transcription and translation? **NusG**
- -important that at exit channel because initiation occurs at exit channel when 16s rRNA recognizes **shine delgarno** on mRNA
- Why would cells bother regulating at the level of translation?
    - Quicker adaptation because of proteins exchanging on ribosome (don't need to wait for new expression of mRNA), reversible
    - Less E cost than degrading proteins they don't need

Experiments

- B-gal assay details
    - SDS and chloroform permeabilize the cells
    - ONPG is a substrate for B-gal that mimics lactose; OTN produces yellow color
    - TSS was originally defined for pdpA by primer extension experiment where you try to extend from mRNA and more RNA-seq data suggested a perhaps longer start site?
- How do you remove purified proteins from anti-VSV-G beads?
    - Elution from beads buffer with SDS and then heat at 65C. This breaks the bond between the antibody and antigen
    - OR you can elute with glycine and a low pH buffer – this is gentler so you can save the protein functionally
- Why do we need to plate different cell densities for population analysis?
    - To identify if there is a heterogeneous population
    - You should plot the x-axis with antibiotic concentration and the y-axis with CFU/mL. A steep curve means homogenous population, a gradual curve means heterogeneous population because there are subpopulations that survive at higher concentrations of the antibiotics
    - Also want to keep number of cells plated less than 10^7 to prevent patch growth of susceptible cells
- Details of transposon mutagenesis
    - Blue/white screening by plating on X-gal plates
    - Bfr promoter, pdpA UTR, fused to LacZ; electroporate in mariner transposon and plate on X-gal
    - If colonies are white (or at least less blue) then the transposon inserted somewhere necessary for efficient translation of pdpA. Use arbitrary PCR to amplify this insert then sequence to find which genes it is.
    - If we don't find that the UTR is sufficient, can we still do this by fusing the first few hundred base pairs to the lacZ gene
    - When do you use Tn-seq – higher throughput so makes more sense for selection like if your transposon has KanR gene – depends on number you are expecting, so >300 or so
    - Using the E. coli lacZ gene
- Details of in vitro translation assays
    - Compare to bfr translation in the same reaction
    - This will account for differences in ribosome numbers as well as other differences in transcription/translation efficiency

    - All of the proteins involved are E. coli proteins, but should be able to use ribosomes from other bacterial species according to product information
    - Need to check that rare codons that would influence E.coli aren't present
- Deletion of rpsU in s. aureus
    - Will probably want to purify ribosomes and make sure they are functional
    - Temperature sensitive origin is active in staph aureus, while constitutive origin is active in E. coli
    - RN4220 has deletions making it restriction deficient
    - At all times in E. coli and RN4220, grow at 30C or 37C. Only when selecting on tet in HG003 do we want to grow at 42C because then only the ones incorporated into the genome will live, as the plasmid will be inactive and tetR won't be expressed
    - Cross out the plasmid by subculturing in liquid media and cross-patching to tet plates
- How does arbitrary PCR work?
    - One primer matches the transposon, one primer is randomized (NNNN)
    - Then do another PCR further downstream of both of these, sharing homology with the randomized primer
- How does Tn-seq work?
    - Your transposon has a cut site that cuts like 20 or so bps away from the actual transposon, so you can digest your genomic DNA and it'll give you small piece of the genomic DNA outside of your transposon
    - Put on adapters so you can PCR amplify then do next gen sequencing
    - On POOLED mutants – can make sure that your entire genome is covered. If you don't get any at a particular region, it indicates that that was an essential gene?
- How does RNA seq work?
    - For my experiment, I'll do it on wild-type and drpsU cells
    - First isolate RNA, deplete ribosomal RNA, fragment the RNA, and generate single stranded cDNA
    - Generate library from illumina by ligating adapters and then next gen sequencing