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Research Plan

Outer Membrane Vesicle and Tube Formation in *Francisella*

A. Rationale:

Outer membrane vesicles and tubes (OMVT) are spherical shaped structures extending from the outer membrane of Gram-negative bacteria. *Francisella tularensis*, is the bacteria that produces OMVT in large quantities. OMVT research is a growing topic due to the discovery of their ability to spread harmful proteins and virulence factors to host cells (Schwechheimer and Kuehn, 2015). The problem for scientists is that little is known about the mechanisms by which OMVT are produced. Further studies on OMVT revealed the presence of hypo-vesiculating genes that led to the production of less OMVT than the wild type (WTU112) strain (McCaig, et al., 2013). Cryoelectron tomography also revealed the formation of bulbs at the sites of OMVT production, believed to be a site of carbon metabolism responsible for OMVT formation. Both *FumA* and *FTN_1037* were pulled from the genetic screen since they expressed the strongest phenotypes of all the hypo-vesiculating genes. This means they are more likely to be expressed in the bulb where the tubes are forming, suggesting their involvement in OMVT formation. Developing an understanding of the mechanisms of tube production could help in preventing OMVT from spreading virulence factors and disease in bacteria. This would enable scientists to regulate their interactions with cells in bacterial communities by reducing their formation once the mechanisms are known. By determining which genes expressed in the bacteria are more involved in the OMVT mechanisms, scientists can begin to target that gene to manipulate it to produce less OMVT. It can also lead to the development of targeted treatment for those infected with tularemia and diseases caused by OMVT production in other bacteria.

B. Hypothesis(es), Research Question(s), Engineering Goal(s), and Expected Outcomes:

Research Question: Can hypo-vesiculating genes be found inside a bulb at the site of OMVT production? What does the localization of *FumA* and *FTN_*1037 indicate about their roles in the mechanisms by which *Francisella* forms OMVT?

Expected Outcomes and Hypothesis(es): It is hypothesized that the fluoresced genes of *FumA* and *FTN_1037* will be present in the same area as the bulb where the tubes are forming. Therefore, the tubes are more likely to be involved in the mechanisms by which OMVT are formed. This is expected since both genes are hypo-vesiculating strains. This means there should be less OMVT grown inside the bacteria when compared to the wild type. *FumA* will reduce OMVT formation by targeting the carbon metabolic activity within the bulb, preventing tubes from forming. *FTN_1037*'s role is unknown but will be able to reduce OMVT formation by targeting some component of the bulb.

C. Procedures, Risk and Safety, Data Analysis, and Discussion of Results and Conclusions

The experiment will be performed at Stony Brook University in the lab of Professor David Thanassi and under the direct supervision of lab assistant Maheen Rashid.

(i) Procedures:

A gradient will be used during PCR to determine the best annealing temperatures. All PCR will be run at 35 cycles with a pre-denaturing step and a post-elongation step at 1 cycle each. Established protocols will be used for time of cycles.

Amplification of genomic DNA

- The *FumA* and *FTN_1037* genes will be amplified from U112 (Wild Type strain) genomic DNA through lab PCR protocols.
- *FumA* should be expressed at 1500 and *FTN_1037* at 1395 bp after being run on a 1.8% agarose gel.
- The bands on the gel will be compared to a 1kb DNA ladder for the properly sized bands and extracted from a gel with the Qiagen QIAquik® Gel Extraction Kit.
- The Qiagen Qiagen QIAquik® Gel Extraction Kit will be used for cleaning DNA for best concentration yields from the nanodrop to ensure there is enough of each gene.

Amplification of *gfp* from Vector

- The *gfp* will be amplified from the vector, *pFNLTP6-gfp*, with specific forward and reverse primers analogous to the DNA sequences for the genes and *gfp* used.
- *Gfp* will run on a 1.8% agarose gel, and is expected to be expressed at 750 bp.
- The bands will be extracted and purified with the same kits as the genes.

Overlap of genomic DNA with *gfp*

- Each gene will be combined in a mastermix with *gfp* at a 1:1 ratio of gene to *gfp* through the PCR program.
- The overlap for each gene will use different forward primers to obtain the desired sequences.
- The *FumA-gfp* overlap (insert) should be expressed at 2250 bp and the *FTN_1037-gfp* overlap (insert) should be expressed at 2145 bp.

Restriction digestion and ligation

- Both the vector, *pFNLTP6-gfp*, and the inserts contain BamHI sites that will be cut by the BamHI-HF enzyme (which will be referred to as Bam H)
- The restriction digestion with BamH was performed separately on the vector and insert with 5 µg of DNA, forming the vector backbone without the *gfp* of the vector.
- Antarctic phosphatase treatment will be performed according to NEB protocol, on the digested vectors and inserts to prepare them for the ligation.
- Qiagen MiniElute® PCR Purification kit will be used to recover the plasmid.
- The ligation will be performed at 1:3 vector to insert ratio attaching the insert to the vector backbone at the BamH sticky ends. This will form the plasmids that were ready to be transformed into *E.coli*.

Transformation into *E.coli*

- Using standard bacterial transformation techniques, the ligated plasmids will be plated onto LB+Kan plates since the vector contains the gene for kanamycin resistance.
- Two plates will serve as controls to the experimental plate: the Self Ligation, which will ensure the vector did not close in on itself with no insert present and the No Ligase, which will ensure there was no colony growth without the ligase enzyme.
- DH5α heat shock protocol will be used with NEB5- α cells with a 1 minute heat shock for the plasmids for their transformation into *E.coli*. Standard bacterial transformation protocols will be utilized.
- The plasmid must be transformed into *E.coli* before *F. novicida* to remove the sites that are cut by enzymes in *Francisella*, allowing the plasmid to readily enter the bacteria.

Transformation into *Francisella*

- Colonies that grow on the ligation plates will be screened to determine whether or not the ligated *pFNLTP6-FumAgfp* plasmid was successfully transformed.
- To do this, the plasmid will be isolated from *E.coli* with a Qiagen Miniprep Kit according to protocol and digested with BamH to compare the sizes of the products. The desired products will include the vector backbone (7200 bp) and *FumA-gfp* insert (2250 bp).
- 100 µL of the plasmid will be sent out for sequencing to confirm the plasmid contained the DNA sequence of the *FumA-gfp* insert.
- A Qiagen MidiElude®Kit will be used to increase the DNA concentration to be electroporated.
- Electroporation will be performed using standard electroporation protocol, to open the wells of the bacteria through an electric shock for the plasmid to enter through the membrane.
- Electroporated cells will be plated on chocolate agar *Francisella* MFN245 plates and kept in a *Francisella* 37°incubator.

Mutant Genes from Genetic Screen:

1. *FumA* – Gene involved in carbon metabolism from stored genomic DNA (U112)
2. *FTN_1037* – Gene whose role is unknown from stored genomic DNA (U112)
3. *Gfp* – Fluorescent tagged protein taken from the pFNLTP6-gfp vector

(ii) Risk and Safety:

- Before experimentation, certain risks, such as, ingestion, inhalation, or any other dangerous contact with hazardous chemicals associated with experiments will be assessed.
- During experimentation, gloves, lab coat and closed-toed shoes will always be worn to prevent skin contact with chemicals being used.
- Flammable chemicals will be handled far from an open flame and under a fume hood. Flammable chemicals are stored in designated cabinets.
- Chemicals and other biological material will be disposed of in proper waste containers, including biohazard waste, or disinfected before disposing.
    - Take precautions when handling Ethidium Bromide (gloves, goggles and apron). Dispose in designed safety container. Never look at the gel on the UV light box without the protective shield and protective eyewear.
    - Bacteria will be placed in 10% bleach before disposing in the designated waste container. (The *Francisella* strain to be used is a BSL-1).
    - All chemicals in Qiagen kits are deemed safe for proper laboratory disposal.
- Caution when handling scalpel, hold the blade down and keep safety cap when not in use. (this procedure will be performed by the mentor).
- Use caution when working with the fluorescent microscope and electroporation device, follow manufacturers guidelines for proper usage.

(iii) Data Analysis

PCR Protocols

- PCR programs will be run following lab protocols to test the DNA concentrations of the genes and *gfp* through every step of the procedure.

- DNA concentrations will be required for determining how much of the gene or *gfp* should be inserted into the master mix for the overlaps and digests.

Gel Electrophoresis

- PCR samples will be run on a 1.8% agarose gel containing ethidium bromide.
- The samples will be compared to 8 µL of a 1 kb DNA ladder for determining gene expression.
- Bands will be imaged under a UV lamp and extracted from the gel at the specified band expression sizes, using a scapel. This step will be performed by the mentor.
- Band expressions will ensure a greater DNA concentration yields and the isolation of both *FumA* and *FTN_1037* from extra PCR products.

Genetic Screening

- A sample of the vector containing the vector with the *FumA-gfp* insert will be sent out to be sequenced to confirm the presence of the *FumA-gfp* sequence.
- This screening will confirm the vector contained the gene to move on to transforming it into *Francisella* and imaging the bacteria.

Fluorescent Microscopy

- Fluorescent microscopy will be used to observe *Francisella* and test for the presence of the GFP-tagged genes.
- The genes will be viewed on the microscope by taking the colonies that grow on the chocolate agar plates and placing them in a liquid LB culture.
- If the genes were identified as being present, their location will be compared to that of the bulb and OMVT production.

- The location of the genes would be a strong indication of their role in OMVT production. If they are located near the bulb and where tubes are produced, it is more likely the gene is involved in the mechanisms of OMVT production.

(iv) Discussion of Results and Conclusions

The results will help in verifying if the genes, FumA and FTN_1037, were successfully tagged with gfp and where it is located within the bacteria. Literature has indicated a decrease in tubes formation with the presence of these two hypo-vesiculating genes than with the wild type U112. Since less OMVT has found to be produced when these genes are present, it is likely that one or both of them interact with OMVT and inhibit some of its growth. The presence of colonies on the chocolate agar plates and the use of fluorescence microscopy imaging will specifically focus on the role FumA has in the mechanisms of OMVT production. FumA is known to be involved in carbon metabolic activity within the bacteria. The bulb is believed to be a site of carbon metabolic activity, which explains why FumA may be expressed there.

FumA-gfp will be grown to colonies on the chocolate agar Francisella plates and identified with fluorescence microscopy. The location of FumA and the amount of OMVT formed will be a good indicator of its involvement in the mechanisms by which *Francisella* produces OMVT. It is possible that this gene reduces OMVT production by producing products during its metabolic activity that conflict with the process during OMVT production. Understanding more about these mechanisms and the genes responsible for promoting OMVT production can provide insight to targeted therapies to reduce its secretions during host cell invasion, reducing disease virulence.

Further research is needed to gain a better understanding of the roles these genes may have in OMVT production. FumA or any other genes with strong phenotypes in OMVT

production could possibly be manipulated for stopping or preventing the spread of virulence factors and pathogens. More research must be done on the mechanisms to officially be able to prevent its OMVT production and virulence spread.

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NO ADDENDUMS EXIST