Mechanism of Outer Membrane Vesicle and Tube Formation in *Francisella*

Theresa Haupt

ABSTRACT

Francisella novicida, a laboratory strain of *Francisella tularensis*, produces outer membrane vesicles and tubes (OMVT) under specific media and growth conditions. OMVT have been found to be associated with several known *Francisella* virulence factors, providing evidence for their role as a special secretion system during host cell invasion. A major focus of OMVT studies is elucidating the mechanisms underlying their production and regulation. To assist this focus, *F. novicida* was analyzed using cryoelectron tomography to acquire detailed structures of these tubes. It appears that a 'bulb-like' structure forms within each tube that pushes the formation of the tubes outwards, suggesting the involvement of many different proteins at this center that could be regulating OMVT production. Using a genetic screen, several hypo-vesiculating mutants were identified as genes with possible involvement in OMVT production. Two of the strongest hypo-vesiculating mutants were found in the genes *FumA* (fumarate hydratase, an enzyme involved in carbon metabolism) and *FTN_1037* (an unknown protein). The purpose of this study was to identify a role for *FumA* and *FTN_1037* in OMVT regulation by analyzing their sub-cellular localization in tube producing bacteria. The goal was to fuse these genes of interest with a green fluorescent protein (*gfp*) and track their localization, with respect to tube formation, using fluorescence microscopy. Both *FumA* and *FTN_1037* have been amplified and fused with *gfp* using overlap extension PCR. The constructs were prepared in the *Francisella* expression vector – pFNLTP6 and transformed into *E. coli* and *F. novicida*. Microscopy was used to verify that the *gfp*-tagged proteins are functional and to study their localization. This study will help acquire more knowledge about the role of *FumA* and *FTN_1037* which would ultimately help in understanding the mechanisms that regulate OMVT production.

INTRODUCTION

Recently, researchers have expressed increased interest in investigating outer membrane vesicles and tubes (OMVT) after discovering their ability to spread virulence factors. OMVTs are spherical shaped structures extending from the outer membranes of Gram-negative bacteria (Rivera and Kuehn, 2016). These structures transport the known antigens, secreted proteins and other virulence factors directly to the host cells after emerging from the bacteria (Schwechheimer and Kuehn, 2015). They are produced in large quantities by *Francisella tularensis*, the bacteria that causes the zoonotic disease tularemia. One hallmark of *Francisella* is its ability to form OMVTs under conditions of amino acid deprivation (Figure 1). It was this observation that suggested regulated OMVT production is dependent upon certain conditions within Gram-negative bacteria. Further studies on the role of amino acid deprivation revealed the presence of hypo-vesiculating (produce less OMVT than wild type (WT)) and hyper-vesiculating (produce more OMVT than WT) mutants that impact the amount of tubes formed. As more evidence on OMVT production by *Francisella* is uncovered, there is still little known about the mechanisms by which they are produced (McCaig, et al., 2013).

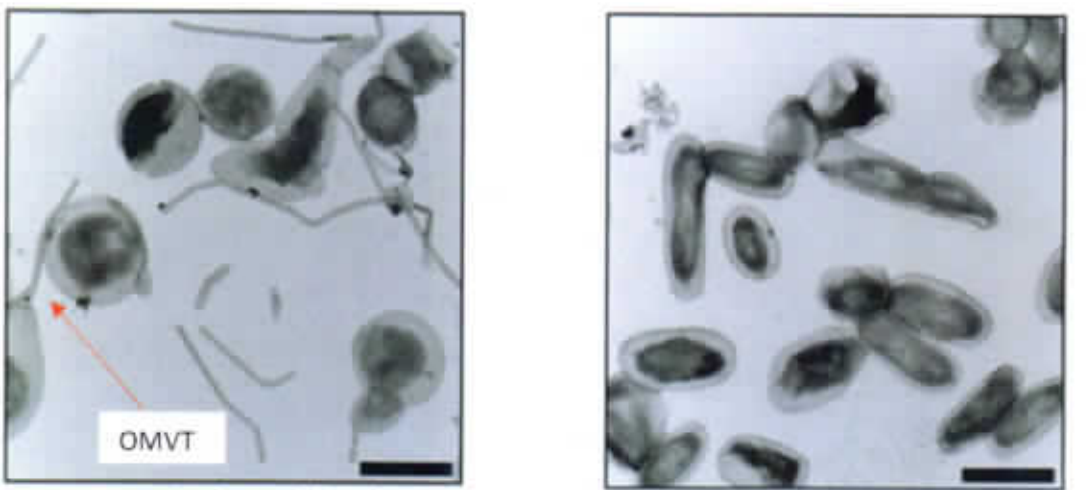

Figure 1. ***F. novicida* produces OMVT in response to amino acid deprivation.** Representative TEM images of OMVT produced by *Francisella* when essential amino acids are depleted, but not produced when amino acids are replete (Sampath, 2018).

F. tularensis is a highly virulent bacteria that is extremely infectious to humans. There have been several subspecies found, one of which is *Francisella novicida*, an experimental strain infectious to mice, but has low virulence to humans (Kingry and Petersen, 2014). Therefore, it was used in this study for lab safety purposes to further observe OMVT production. A genetic screen was performed identifying the hypo-vesiculating mutants and comparing them to the WT strain *F. novicida* U112 (Rashid, M., personal communication). The genes chosen from this screen were *FumA*, a gene involved in carbon metabolism,

and *FTN_1037*, whose role in this bacterium is unknown. Since these are hypo-vesiculating genes, it is possible their presence in the bacteria may be essential to preventing OMVT production (Figure 2).

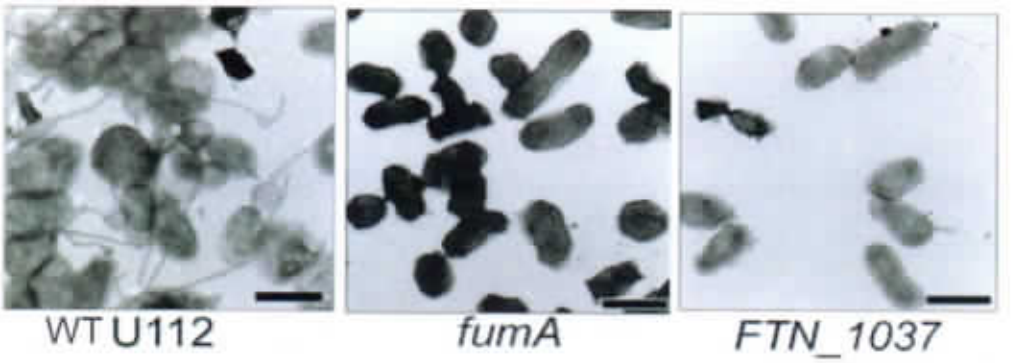

Figure 2. **Genetic Screen leads to identification of hypo-vesiculating genes with significant reduction in OMVT.**
Representative whole bacterial TEM images showing OMVT production in WT U112 and the two hypo-vesiculating genes. *FumA* and *FTN_1037* grow less OMVT under amino acid depletion conditions than the WT.

Cryoelectron tomography (cryo-ET), generated in collaboration with Liu Lab at Yale University, was performed to gather images within the bacteria where tubes formed (Figure 3). These images revealed a bulb-like structure that has never been seen before at the exact site of tube formation. It is speculated that this may be a center of metabolic activity where the genes of the hypo-vesiculating mutants could be involved in OMVT production.

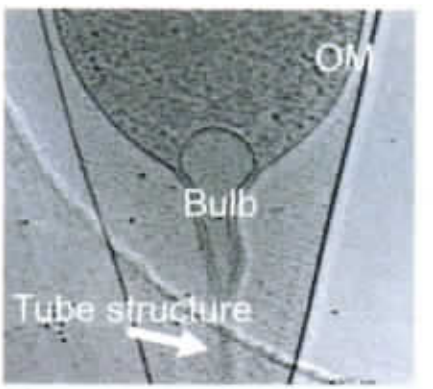

Figure 3. **Bulb found in cryo-ET could contain genes that are involved in OMVT production.**
Cryo-ET image of a bulb shaped structure where tubes have formed in *Francisella*.

The purpose of this experiment was to gain a better understanding of the mechanisms of OMVT production by studying the subcellular delocalization of the genes *FumA* and *FTN_1037*, found in *Francisella*. Acquiring more knowledge on this subject would determine whether these genes are involved in tube formation, providing evidence for their role as a special secretion system during host cell invasion. This would then help in understanding the mechanisms which regulate or control OMVT production, which is important because they are associated with virulence factors. Both *FumA* and *FTN_1037* have expressed the strongest phenotypes of all the genes pulled from the genetic screen. This means they are more likely to be expressed in the bulb where the tubes are forming, suggesting their involvement in OMVT formation.

MATERIALS AND METHODS

The mutants tested were taken from a genetic screen of several mutants prior to this study being performed (Sampath, et al. 2018). The mutants from the screen were identified as either hypo-vesiculating or hyper-vesiculating, but only the four hypo-vesiculating mutants (*FumA*, *TktA*, *FTN_1037*, and *FTN_0908*) were noted for their severe reductions in OMVT production. These mutants expressed a greater possibility of being involved in the mechanisms of OMVT production since their presence leads to less OMVT produced than WT U112, but only *FumA* and *FTN_1037* were tested.

In this study, both genes were overlapped with a protein that encodes for fluorescence (*gfp*) to observe the bacteria through fluorescence microscopy. Each gene was amplified from U112 genomic DNA while *gfp* was amplified from its vector, pFNLTP6-gfp which was derived from the pFNLTP1 plasmid (Maier, et al. 2004).

1. Amplification of genomic DNA

i. PCR Amplification of *FumA*

Table 1. **PCR mixture for the amplification of *FumA* from U112 genomic DNA.**
The PCR mix for amplifying *FumA* included the reverse primer with the sequence for overlapping with the *gfp* forward primer sequence. The mix was separated into 4 PCR tubes of 50 µl.

	50 µl
5X GC Buffer	10.0
Nucleotides	1.0
Forward Primer	2.5
Reverse Primer	2.5
DMSO (100%)	1.5
U112g DNA (61µg/µl)	3.7
Phusion Enzyme	0.5
H2O	28.3

The PCR had an initial temperature of 98°C for 30 seconds for 1 cycle; a **denaturation** temperature of 98°C for 10 seconds, an **annealing** temperature of 48°C for 30 seconds, an **extension** temperature of 72°C for 1 minute and 40 seconds for 35 cycles; and a final temperature of 72°C for 10 minutes for 1 cycle. The amplified genes stayed at 4°C until the next step of the procedure. The gene, *FumA*, amplicon expressed at 1500 base pairs (bp). The forward primer contained the sequence that would later attach to

the BamH site of the vector backbone. The reverse primer contained the sequence that matches the forward primer sequence of *gfp*.

ii. PCR Amplification of *FTN_1037*

Table 2. **PCR mixture for the amplification of *FTN_1037* from U112 genomic DNA.**
The PCR mix for amplifying *FTN_1037* included the reverse primer with the sequence for overlapping with the *gfp* forward primer sequence. The mix was separated into 4 PCR tubes of 50 µl.

	50 µl
5X HF Buffer	5.0
Nucleotides	1.0
Forward Primer	2.5
Reverse Primer	2.5
DMSO (100%)	1.5
U112g DNA (52µg/µl)	3.8
Phusion Enzyme	1.0
H2O	32.7

The PCR had an initial temperature of 98°C for 30 seconds for 1 cycle; a **denaturation** temperature of 98°C for 10 seconds, an **annealing** temperature of 49°C for 30 seconds, an **extension** temperature of 72°C for 1 minute and 30 seconds for 35 cycles; and a final temperature of 72°C for 10 minutes for 1 cycle. The amplified genes also stayed at 4°C until the next part of the procedure. The gene, *FTN_1037*, amplicon expressed at 1395 bp. The forward primer contained the sequence that would attach to the BamH site of the vector backbone. The reverse primer contained the sequence that overlaps with the forward primer sequence of *gfp*.

The PCR program had to be experimented with through a gradient PCR to determine the best annealing temperature for obtaining the gene.

iii. Gel Extraction (followed protocols for the Qiagen QIAquick® Gel Extraction Kit QIAquick® PCR & Gel Cleanup Kit)

Some modifications were made to the protocol found in the kit to obtain higher DNA yields. The gel was dissolved in a water bath for 20 minutes instead of 10 minutes to account for the greater gel mass in each tube. The kit is designed for gels of no greater than 500 grams to fit the entire volumes of the solutions used. Buffer EB from was warmed in the heat bath until being used in the last step to prevent contamination. The gel extractions were performed for the genes, *gfp*, and vector digests.

iv. Mini Elute for DNA Purification ((followed protocols for the Qiagen MinElute PCR Purification Kit)

DNA concentrations were measured with the NanoDrop following the steps listed on the computer software. The DNA was purified due to the NanoDrop yielding low concentrations. The only modification made from the kit protocol was that the buffer EB was again warmed to prevent contamination.

2. Amplification of *gfp* from vector

Table 3. **PCR mixture for the amplification of *gfp* from pFNLTP6-gfp.**
The PCR mix for amplifying *gfp* included the forward primer with the sequence for overlapping with the *FumA* and *FTN_1037* reverse primer sequence. The mix was separated into 5 PCR tubes of 50 µl.

	50 µl
5X HF Buffer	10.0
Nucleotides	1.0
Forward Primer	2.5
Reverse Primer	2.5
pFNLTP6-gfp	0.5
Phusion Enzyme	0.5
H2O	33.0

The PCR had an initial temperature of 98°C for 30 seconds for 1 cycle; a **denaturation** temperature of 98°C for 10 seconds, an **annealing** temperature of 51°C for 30 seconds, an **extension** temperature of 72°C for 1 minute for 35 cycles; and a final temperature of 72°C for 10 minutes for 1 cycle. The *gfp* protein amplicon was expressed at 750 bp. The PCR was performed on 5 tubes with 50 µl of the mix to be imaged on a gel. The forward primer contained the sequence that would attach to the reverse primer of *FumA* and *FTN_1037*. The reverse primer contained the reverse primer sequence that overlaps with the forward primer sequence of the other BamH site. This protein was taken from the vector, pFNLTP6-gfp.

3. Overlap of genomic DNA with *gfp*

The genes and *gfp* were combined in a mastermix for PCR. The ratio of the gene to *gfp* was a 1:1 ratio. The program for both genes was the same, starting with of 98°C for 2 minutes for 1 cycle; followed by a **denaturation** temperature of 98°C for 15 seconds, an **annealing** temperature of 50°C for 30 seconds, and an **extension** temperature of 72°C for 2 minutes for 10 cycles; and a final temperature of 72°C for 2 minutes for 1 cycle. After the 10 cycles, 2.5 µl of primers were added to each sample and ran on another

PCR with an annealing temperature of 66°C for 30 cycles. The only difference in the second PCR was that *FumA* and *FTN_1037* had different forward primers added. The *FumA-gfp* overlap was to be expressed at 2250 bp and the *FTN_1037-gfp* overlap was to be expressed at 2145 bp.

Figure 4. **Diagram of the *FumA-gfp* and *FTN_1037-gfp* overlaps after PCR amplification.** The genes were tagged with *gfp* by overlapping the reverse primer of the gene with the forward primer of *gfp*.

4. Restriction Digestion and Ligation (Standard Restriction Digest and Ligation Protocol)

i. BamH restriction sites were used throughout the procedure

The overlap for both genes joins with the vector backbone at BamH sites where the enzyme (BamHI-HF) cuts the vector. The restriction digestion was modified to implement 5 µg of DNA instead of 1 µg. The vector and insert were digested separately, both with 2 µl of the BamH enzyme and 5 µl of 10X Cut Smart buffer. The vector backbone is formed in this step as the enzyme removes the *gfp* from the *pFNLTP6-gfp* at the BamH sites. The insert can then replace the *gfp* as it contains the sticky ends to combine with the BamH sites of the vector backbone.

ii. Antarctic Phosphatase Digest (followed protocols for Dephosphorylation of 5'-ends of DNA using Antarctic Phosphatase (NEB #M0289))

This AP treatment was performed on the digested vector and insert for both genes to prepare them for the transformation and check DNA concentrations. With this treatment, 3 µl of water, 1 µl of the Antarctic Phosphatase enzyme, and 6 µl of the buffer were added to the 50 µl digest. The treated samples were then left in an 37°C incubator for 30 minutes and heated at 80°C for 5 minutes.

iii. Vector Insert Ligation (followed standard protocols for Insert + Vector DNA Ligation)

Before the plasmid can be transformed into *F. novicida*, it has to go through *E. coli*, to MFN245, and end in U112 because *F. novicida* U112 has a restriction barrier that breaks up the extra DNA. The ligation to complete the process depicted in figure 5 was done at a 1:3 vector to insert ratio. The other ratio attempts were a 1:10 and a 1:6, but both of these ligations yielded no colonies.

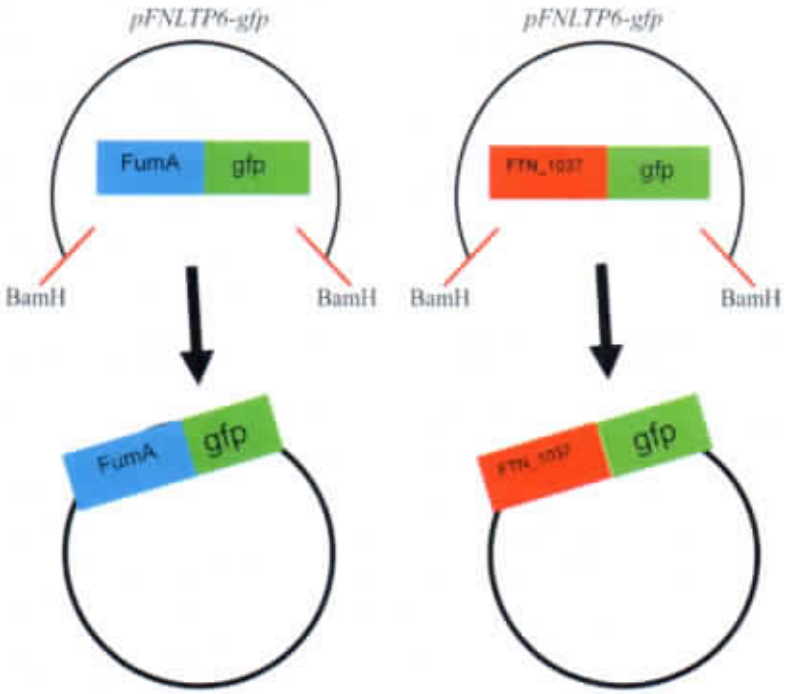

Figure 5. **Diagram of vector backbone indicating BamH sites and vector containing the insert after it has been digested and ligated.**
(Before) The vector backbone is produced by using BamH enzyme to cut at the BamH sites where the insert (overlap of genes with *gfp*) will be located. (After) The vector was ligated, forming the plasmid that is ready to be transformed into *E. coli* and then *F.novicida*.

5. Transformation into *E. coli*

i. Plating of Ligated Plasmids

The digested vector and inserts, after going though AP treatment, were plated onto LB + Kan plates because the vector contains the gene for kanamycin (Kan) resistance. Along with the experimental ligation plate, two other plates served as controls. The self-ligation control served as a test to ensure the vector did not close in on itself when there is no insert present. If colonies do grow on this plate, that suggests the insert did not combine with the vector backbone. The no-ligase control served as a test to ensure no colonies would grow without the ligase enzyme. The plates were left overnight at 37°C to check for colony growth the next day.

ii. DH5α Heat Shock Protocol (followed Standard Transformation Protocol Using Heat Shock)

The *E. coli* transformation was changed from DH5α to NEB5-α cells because the DH5α cells were not competent enough for growing colonies. It was also determined through trial and error that a 1-minute heat shock was more efficient than a 2-minute heat shock.

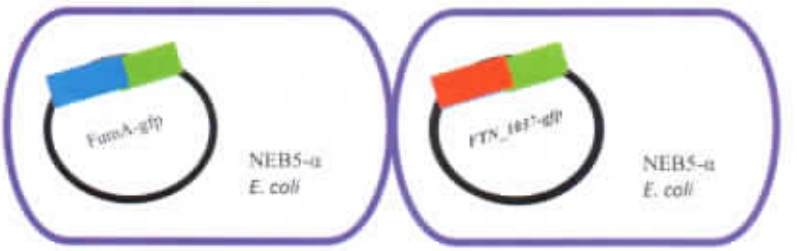

Figure 6. **Transformation of the plasmids containing *FumA-gfp* and *FTN_1037-gfp* overlaps into *E. coli*.**
The left figure displays a model of the *FumA* overlap successfully expressed in *E. coli* while the right one displays the *FTN_1037* overlap successfully expressed in *E.coli*.

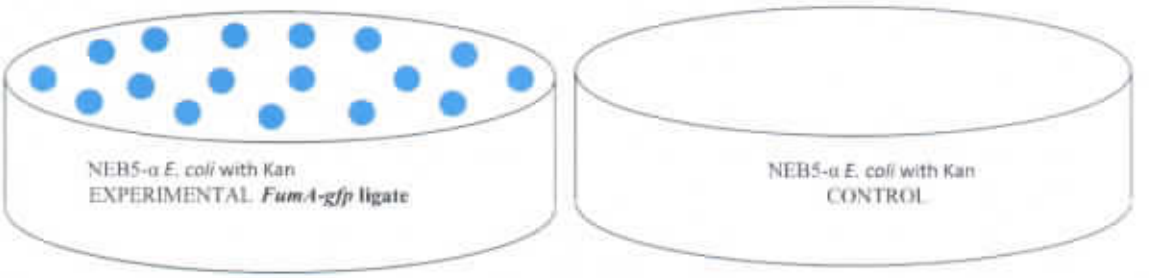

Figure 7. **Experimental and control plates after *FumA-gfp* ligation was grown overnight.**
The plate on the left was the tested ligation plate for the *FumA-gfp* vector transformed into *E.coli*. The presence of colonies on this plate and not the control plates mean the transformation was succesful.

6. Transformation into *Francisella*

i. Screening of Colonies

At this point, only the plasmid containing *FumA* was tested, as complications arose with the *FTN_1037* ligation. The few colonies that grew with *FTN_1037* have not yet been screened and imaged. The colonies that grew on the *FumA-gfp* ligation plates were screened the next day to test whether the they were the desired ligated product. They could have been either the ligated vector, pFNLTP6-FumA-gfp, or the undigested vector. The ligated vector needs to be present for the plasmid to be transformed into *Francisella*. 17 colonies from the plate were inoculated in 5 mL LB and 50 µl kanamycin for screening.

ii. Mini Prep Protocol

The plasmid was isolated with a Mini Prep protocol and digested with BamH to compare the sizes of the products. The pFNLTP6-*FumA-gfp* bands would be expressed at 2250 bp (*FumA-gfp* that was released

from the vector after the digest) and 7200 bp (vector backbone without insert). The undigested vector bands would still have the vector backbone, but the *gfp* band would be 750 bp. The pFNLTP6-*FumA-gfp* bands confirmed the desired plasmid was isolated. The digest was elongated from 2 hours to being left overnight (~10 hours) for better yield.

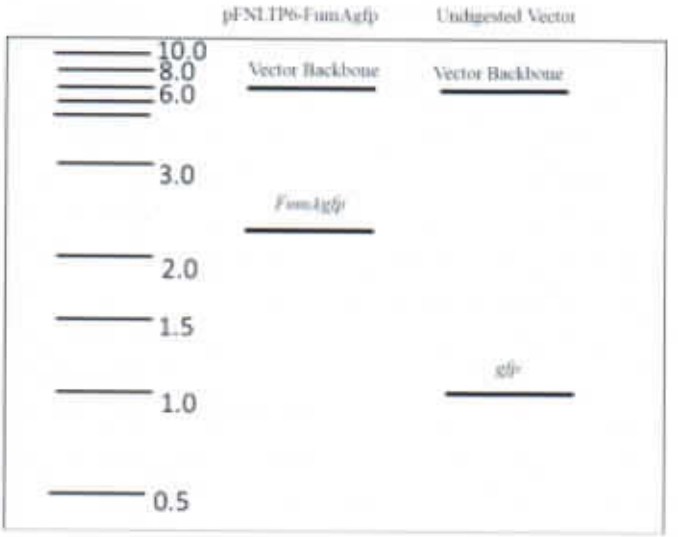

Figure 8. **Theoretical Gel showing the band sizes for pFNLTP6-*FumA-gfp* vector and an undigested vector against a 1Kb ladder**.
The gel for the pFNLTP6-*FumA-gfp* has a *FumA-gfp* band expressed at ~2250 bp and the vector backbone at 7200 bp, the *gfp* alone expresses at ~750 bp

iii. Sequencing Confirmation

100 µL of the plasmid was sent out to confirm sequencing of the plasmid according to the sequence lists for *FumA*, *FTN_1037*, and *gfp*. The sequencing was done to check that the *FumA-gfp* or *FTN_1037-gfp* overlaps were present in their respective plasmids.

iv. Midi prep of plasmid (followed protocol for GenElute™ HP Plasmid Midiprep Kit)

Midi Elude was used to hold 100 µg of the plasmid from a 50 mL overnight culture instead of 10 µg from a 5 mL overnight. Once isolated, the plasmid concentration was checked with the nanodrop, which read a concentration of 277 ng/µL. The plasmid was further concentrated with the protocol for concentrating plasmids using pellet paint. The higher concentrated plasmid was 1954 ng/µL, close to the 2 µg required for electroporation.

v. Electroporation (modified from Lo Vullo protocol (2006).

The plasmid had to be transformed into MFN245 before being inserted into *F. novicida* to remove the cutting sites and enable the vector to readily enter the bacteria. The general electroporation protocol can be seen in Figure 9.

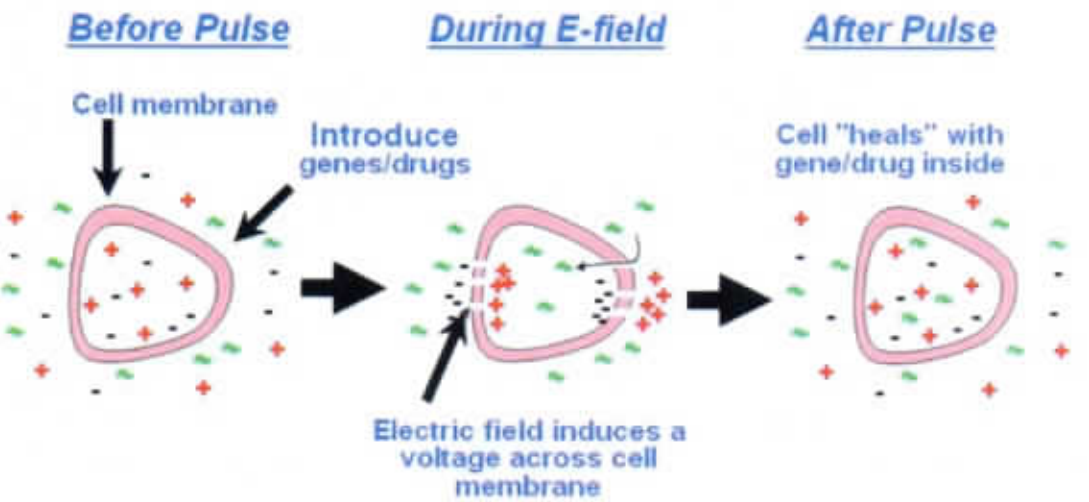

Figure 9. **Diagram of the electroporation as it occurs inside any bacterium.**
Electroporation produces an electric shock that temporarily opens up the walls of bacteria to allow a plasmid to enter. For this study's purposes, the bacterium was *Francisella* and electroporation aided in the transformation of the plasmid containing *FumA-gfp* into the bacterial cell ("Bacterial Transformation," 2014).

Figure 10. **Transformation of the plasmids containing *FumA-gfp* and *FTN_1037* overlaps into *Francisella*.**
The left figure displays a model of the *FumA* overlap successfully expressed in *Francisella* while the right one displays the *FTN_1037* overlap.

The electroporation sends an electric shock to the bacteria to temporarily open the wells for the plasmid to enter through the membrane. The colonies were washed in 12.5 mL of sucrose, resuspended and then centrifuged for 10 minutes. The cells for electroporation were followed using the protocol, incubated for about three hours, and plated on chocolate agar *Francisella* plates. There were two plates set up, a negative control and an experimental MFN245 plate, each with 150 µg of kanamycin. The plates were

stored in a *Francisella* incubator to grow the colonies that would be looked at during the fluorescence microscopy.

7. Fluorescence Microscopy

i. Day Culture Prep

Colonies from the experimental chocolate agar plates were grown in a culture overnight and stored in -80 incubator specialized for working with *Francisella*. The day culture was made the next morning with 1 mL of the overnight, 25 mL BHI medium, and 15 µL Kan. Before the overnight was added, the flask containing BHI and Kan was placed in a CO_2 incubator for 20 minutes since *Francisella* needs CO_2 to grow. The day culture was shaken in an incubator for about 2 hours until an OD of 0.8 was reached. Once the OD reached around 0.8, the microscopy slides were made.

ii. Microscopy – Slides and Imaging

Fluorescence microscopy was used to observe the bacteria and view the green fluorescence emitted by the mutants to determine if they are expressed near OMVT production or the bulb. Fluorescence microscopy magnification is not able to see the tubes formed by *Francisella*, as it is too small to be seen at 200 nm. The microscope can be used to detect the *gfp* and capture an image of the fluoresced genes.

RESULTS

All gel images were recorded and annotated for reviewing band sizes to confirm the proper bands were present. The first gels were performed for the amplified genes of *FumA* and *FTN_1037*. These gels, shown in figures 11 and 12, indicate the amplicons of the genes to be extracted and overlapped with *gfp*.

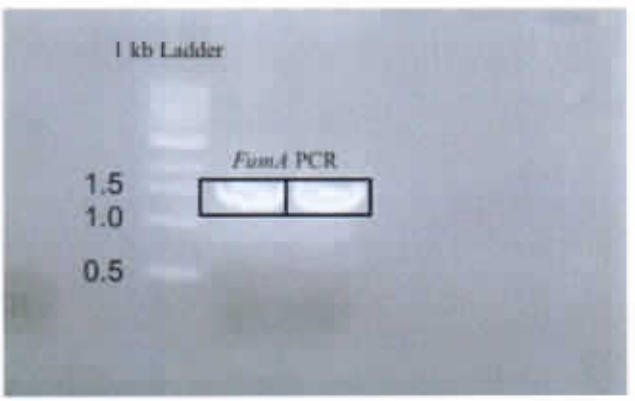

Figure 11: Amplification of *FumA* from using PCR.
The gene for *FumA* was amplified through a PCR and ran on a polyacrylamide gel to be extracted at 1500 bp and purified. The purified gene was stored to be used in the *FumA* and gfp overlap.

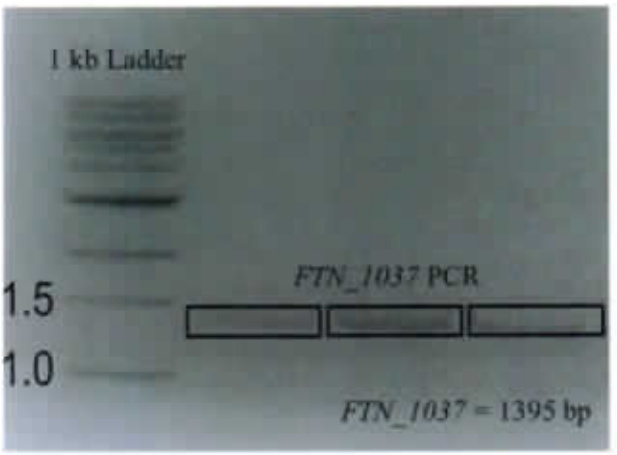

Figure 12: Amplification of *FTN_1037* from using PCR.
The gene for *FTN_1037* was amplified through a PCR and ran on a gel to extract the bands at 1395 bp. The bands were then purified and stored to be used in the overlap with *gfp*.

Similar to the amplification of *FumA* and *FTN_1037*, the *gfp* amplicon is depicted in the gel image (figure 13) at the correct band size (750 bp). The gel exemplifies the successful expression of *gfp* from the original vector, *pFNLTP6*.

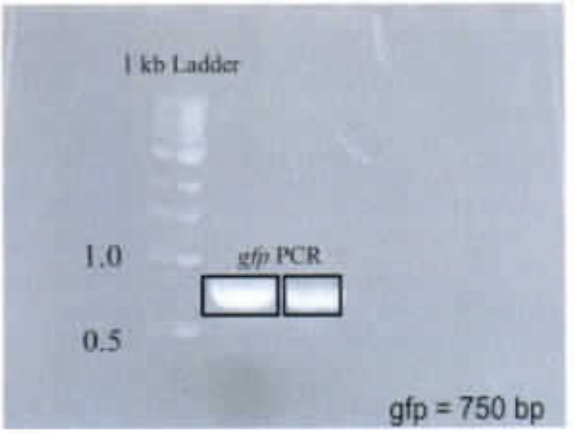

Figure 13: Amplification of gfp from using PCR.
The gfp was isolated from the vector pFNLTP6-gfp and ran on a gel. The bands were then extracted from 750 bp and purified to later be used for the overlap.

Both genes were overlapped with *gfp* through a PCR program that used specific forward and reverse primers. Figure 14 displays the *FumA-gfp* overlap, which yielded the precise bands at 2250 bp. Figure 15 displays the *FTN_1037-gfp* overlap, which also yielded precise bands at 2145 bp. The success of the overlaps for both genes demonstrates a success in the primers used. The forward primer of *gfp* met with the reverse primer of the gene.

Figure 14: *FumA* and gfp overlap.
FumA and gfp were combined through an overlap using forward primer from the *FumA* PCR and reverse primer from the gfp PCR to ensure the DNA strands attached.

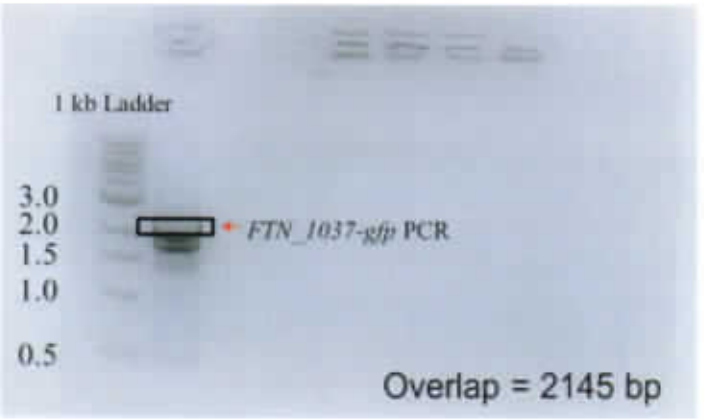

Figure 15: Overlap of *FTN_1037* and *gfp*.
FTN_1037 and *gfp* were combined through an overlap using the forward primer from 1037 and reverse primer from *gfp* to attach the DNA strands.

The restriction digest was performed on the vector, *pFNLTP6-gfp*, to cut the plasmid at BamH sites by removing the *gfp*. This created the vector backbone labeled in figure 16. The inserts, *FumA-gfp* and *FTN_1037* overlaps, were also digested to create the sticky ends that would attach to the BamH sites on the vector backbone. The digest was successful for both genes, but only the gel for *FumA* was included. *FumA-gfp* was ligated into the vector and transformed into *E.coli*. Colonies containing this plasmid grew on the LB + Kan plates.

The expected gel results for the screening of colonies are similar to the expected results of the restriction digest before the ligation is complete. Most of the colonies that were screened contained the undigested vector, which did not release *FumA-gfp*. Only the 17th colony had the desired plasmid, which is depicted in the gel from figure 17.

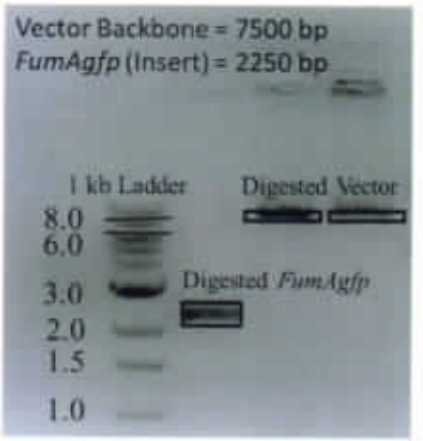

Figure 16: Restriction digest of the pFNLTP6-gfp vector to prepare for ligation.
The pFNLTP6-gfp vector was digested overnight with BamHI-HF enzyme along the BamH restriction sites. This was done to isolate the vector backbone to later insert the *FumA* and gfp overlap in a ligation.

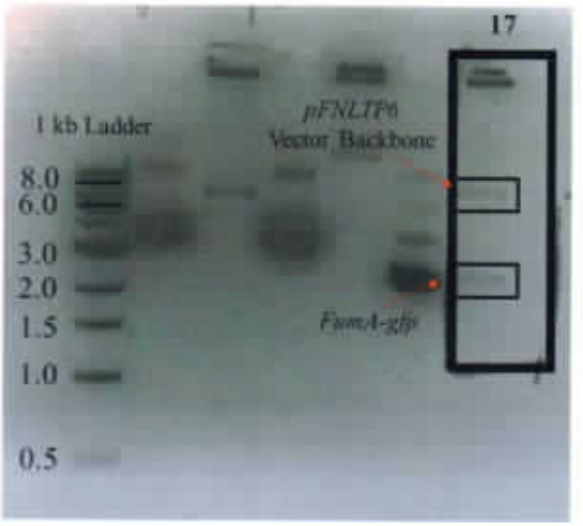

Figure 17: Screening of Colony 17 indicating the only colony to have the desired plasmid.
Colony 17 from the transformed colonies on the *E. coli* plate expresses the bands for the vector backbone and *FumA-gfp* insert, showing the vector is present.

This screening revealed a colony containing the vector and insert with the expected band sizes (vector was at 7500 bp and the *FumA-gfp* insert was at 2250 bp). It was this colony that was transformed into MFN245 and observed with fluorescence microscopy, which revealed the gene within the bacteria. Sequencing of the plasmid from this colony verified that the sequence for the overlap was present.

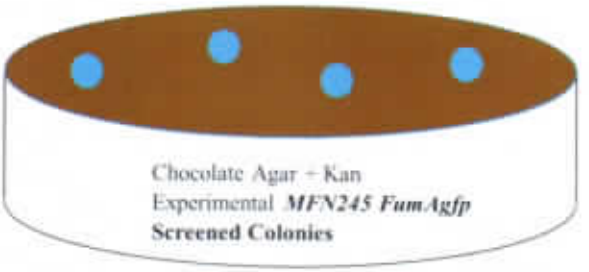

Figure 18: Chocolate agar *Francisella MFN245* plate with *FumAgfp* colonies after.
The pFNLTP6-gfp vector was digested overnight with BamHI-HF enzyme along the BamH restriction sites. This was done to isolate the vector backbone to later insert the *FumA* and gfp overlap in a ligation.

FumA-gfp grew on the chocolate agar plates before being transformed into MFN245. These colonies went through electroporation and were transformed into *Francisella*. The fluorescent microscopy revealed the fluoresced gene, but not the bulb or site of OMVT formation. The problem with fluorescent microscopy is that the magnitude is too small to identify distinct tube production and features. *F. novicida* is at 200 nm, so super-resolution microscopy would have to be performed to see smaller features, such as the bulb. The fluorescent microscopy did indicate that the vector containing *FumA-gfp* was present in the strain when tubes formed.

DISCUSSION AND CONCLUSIONS

The results helped in verifying that the genes, *FumA* and *FTN_1037*, were successfully tagged with *gfp* and then located within the bacteria. However, it cannot be confirmed that the genes were located inside the bulb. It can only be concluded that the genes are present when tubes are formed. Tube formation by the *F. novicida* strain was tested in prior studies and referred to for this data collection. Both the experiment and literature have indicated a decrease in tubes formation with the presence of these two hypo-vesiculating genes than with the wild type U112. Since less OMVT are 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 fluorescence microscopy imaging specifically focuses 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 grew colonies on the chocolate agar *Francisella* plates and was identified with fluorescence microscopy. The location of *FumA* and the amount of OMVT formed is 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. Neither *FumA* nor *FTN_1037* has been located in or out of the bulb, so it is possible either one could play a role. 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. The screening and electroporation protocols still need to be completed for *FTN_1037* to confirm the plasmid is present for that gene as well where it can then be transformed into *Francisella* to observe where the gene is expressed within the bacteria. Once both genes have been seen through fluorescent microscopy, more detailed imaging, such as Cryo-ET can be used to observe the distinct features of the OMVT and fluoresced genes. More detailed imaging could include super-resolution microscopy or the use of more advanced equipment. The location and amount of tubes formed would need to be compared between the two genes to determine which one is more involved in OMVT mechanisms. Based off *FumA's* role in carbon metabolism and the data collected on it, more tests should be done on *FumA* to observe the biological processes occurring that are not visible to the human eye. *FumA* or any other genes with strong phenotypes in OMVT production could be manipulated for stopping or preventing the spread of virulence factors and pathogens.

Bibliography

Bacterial Transformation: Electroporation. (2014). *Society for Mucosal Immunology*. Retrieved from http://www.socmucimm.org/bacterial-transformation-electroporation/.

Carbon Metabolism – Biosystems. (n.d.). In *Kyoto Encyclopedia of Genes and Genomes*. Retrieved from https://www.ncbi.nlm.nih.gov/biosystems/815530?Sel=geneid:31915 34#show=genes.

Cecil, J. D., Sirisaengtaksin, N., O'Brien-Simpson, N. M., & Krachler, A. M. (2019). Outer Membrane Vesicle-Host Cell Interactions. *Microbiology spectrum, 7*(1), 10.1128/microbiolspec.PSIB-0001-2018. doi:10.1128/microbiolspec.PSIB-0001-2018

Elhenawy, W., Bording-Jorgensen, M., Valguarnera, E., Haurat, M.F., Wine, E., Feldman, M.F. (2016). LPS Remodeling Triggers Formation of Outer Membrane Vesicles in *Salmonella*. *mBio*, 7(4), e00940-16. DOI: 10.1128/mBio.00940-16

FumA (2019). NCBI. Retrieved https://www.ncbi.nlm.nih.gov/gene/3191534

Gallagher, L. A., McKevitt, M., Ramage, E. R., & Manoil, C. (2008). Genetic dissection of the *Francisella novicida* restriction barrier. *Journal of Bacteriology, 190*(23), 7830–7837. doi:10.1128/JB.01188-08

Hollis, D.G., Weaver, R.E., Steigerwalt, A.G., Wenger, J.D., Moss, C.W., and Brenner, D.J. (1989). *Francisella philomiragia* comb. nov. (formerly *Yersinia philomiragia*) and *Francisella tularensis* biogroup *novicida* (formerly *Francisella novicida*) associated with human disease. *Journal of Clinical Microbiology, 27*(7), 1601-1608.

Jan A. T. (2017). Outer Membrane Vesicles (OMVs) of Gram-negative Bacteria: A Perspective Update. *Frontiers in Microbiology, 8,* 1053. doi:10.3389/fmicb.2017.01053

Kingry, L.C. and Petersen, J.M. (2014). Comparative review of *Francisella tularensis* and *Francisella novicida*. *Frontiers in Cellular Infection and Micorbiology, 10*, 33-89.

Kulp, A., and Kuehn, M.J. (2010). Biological Functions and Biogenesis of Secreted Bacterial Outer Membrane Vesicles. *Annual Review of Microbiology, 64*(1), 163-184.

Larsson, P., Oyson, P.C., Chain, P., Chu, M.C., Duffield, M., Fuxelius, H.H.., … Titball, R.W. (2005). The complete genome sequence of *Francisella tularensis*, the causative agent of tularemia. *Nature Genetics, 37*(2), 153-9.

Lo Vullo. (2006). *Francisella tularensis*. Electroporation Protocol. *Microbiology*, 3425-3435.

Maier, T. M., Havig, A., Casey, M., Nano, F. E., Frank, D. W., & Zahrt, T. C. (2004). Construction and characterization of a highly efficient *Francisella* shuttle plasmid. *Applied and Environmental Microbiology, 70*(12), 7511–7519. doi:10.1128/AEM.70.12.7511-7519.2004

Malabirade, A., Habier, J., Heintz-Buschart, A., May, P., Godet, J., Halder, R., … Fritz, J. V. (2018). The RNA Complement of Outer Membrane Vesicles from *Salmonella enterica Serovar Typhimurium* Under Distinct Culture Conditions. *Frontiers in Microbiology, 9*, 2015. doi:10.3389/fmicb.2018.02015

McCaig, W.D., Koller, A., and Thanassi, D.G. (2013). Production of Outer Membrane Vesicles and Outer Membrane Tubes by *Francisella novicida*. *Journal of Bacteriology, 195*(6), 1120-1132.

Petersen, J. M., and Molins, C. R. (2010). Subpopulations of *Francisella tularensis* ssp. *tularensis* and *holarctica*: identification and associated epidemiology. *Future Microbiol.* 5, 649–661.

Rivera, N.O. and Kuehn, M.J. (2016). Environmentally Controlled Bacterial Vesicle-Mediated Export. *Cell Microbiol, 18*(11), 1525-1536.

Sampath, V., McCaig, W.D., and Thanassi, D. G. (2018). Amino acid deprivation and central carbon metabolism regulate the production of outer membrane vesicles and tubes by *Francisella*. *Molecular Microbiology, 107*(4), 523-541.

Schwechheimer, C. and Kuehn, M.J. (2015). Outer-membrane vesicles from Gram-negative bacteria: biogenesis and functions. *Nat Rev Microbiol, 13*(10), 605-619.

Toyofuku, M., Nomura, N., and Eberi, L. (2019). Types and origin of bacterial membrane vesicles. *Nature Reviews Microbiology, 17*(9), 13-24.

Wessel, A.K., Liew, J., Kwon, T., Marcotte, E.M., and Whiteley, M. (2012). Role of *Pseudomonas aeruginosa Peptidoglycan*-Associated Outer Membrane Proteins in Vesicle Formation. *Journal of Bacteriology, 10*, 1253-1265.

Yonezawa, H., Osaki, T., Kurata, S. et al. Outer Membrane Vesicles of *Helicobacter pylori* TK1402 are Involved in Biofilm Formation. *BMC Microbioly, 9*, 197. doi:10.1186/1471-2180-9-197