5-2020

Amplification and Potential Transformation of Human Syntaxin-17 into Model E. coli

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Amplification and Potential Transformation of Human Syntaxin-17 into Model *E. coli*

by

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A Thesis
Submitted to the Honors College of
The University of Southern Mississippi
in Partial Fulfillment
of Honors Requirements

May 2020

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Abstract

Membrane fusion is key to organism homeostasis and occurs when a transport vesicle fuses with a target compartment, merging the two membranes into one while releasing the contents of the transport vesicle into the target compartment. This process is controlled by SNARE proteins. Syntaxin-17 protein plays a crucial role in the fusion of the autophagosome membrane with the lysosome membrane, allowing for degradation of misfolded intracellular components and pathogens. Intriguingly, Syntaxin 17 has been identified as a target of herpesvirus tegument proteins, although the pathological significance of the Syntaxin 17 and tegument protein interaction is unclear. As a first step towards characterizing the biochemical and functional interaction between the tegument proteins and the target SNARE, this study aimed to amplify and introduce the Syntaxin 17 cDNA into a model *E. coli* for production and purification of Syntaxin 17 protein. Amplified insert and vector cDNA of the correct size were obtained and verified via gel electrophoresis. Future research should focus on the transformation of the chimeric plasmid into competent *E. coli*. If isolates with the correct size insert were obtained, the next steps would be sequencing to confirm absence of mutation and proceed to affinity column protein purification to obtain the pure recombinant proteins.

Keywords: Membrane fusion, Syntaxin 17, SNARE, Degranulation, Tegument protein, Autophagosome

Acknowledgements

I would like to thank my thesis advisor, Dr. Hao Xu, for allowing me into his research laboratory and teaching me what genuine research is. This project would have been impossible if it were not for his continual guidance and support throughout the research process. Thank you for not only allowing me to work in your laboratory, but also allowing me to be a part of the family. I will forever be grateful for his continual push for me to understand all aspects of my research and to not just show up and follow directions on a protocol.

I would like to offer my sincerest gratitude to graduate students Pratikshya Adhikari and Tolulope Ayo for the countless hours of training and assistance they provided while answering my ceaseless stream of seemingly simple questions. Also, I wish to express my gratitude to fellow undergraduates Amanda Ray and Logan Ryals. You have always been a lifeline for me during this project and constantly answered my calls for help, no matter how late at night they were.

Finally, I wish to thank my family and friends for understanding and putting up with my many late nights spent in the laboratory and pushing me to perform the best research that I could.

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List of Abbreviations

CFU	Colony Forming Unit
HSV	Herpes Simplex Virus
KSHV	Kaposi Sarcoma Herpes Virus
MBP	Maltose Binding Protein
SNAP29	Synaptosomal-Associated Protein 29
SNARE	Soluble N-Ethylmaleimide-Sensitive Factor Attachment Protein Receptor
Stx-17	Syntaxin-17
VAMP	Vesicle-Associated Membrane Protein

Chapter 1: Introduction

Mast cells are key to the detection of and host response to pathogenic infections (Viret *et al.*, 2018). These cells are resident immune cells that inhabit tissues such as the connective tissue that underlies epithelial and mucosal tissue (Krystel-Whittemore *et al*., 2016). They are referred to as granulocytes because they have granules in their cytoplasm that contain bioactive molecules that can cause inflammation and an immediate allergic response (Pejler *et al.*, 2014). The cells not only effect allergic response but are also responsible for initiation and regulation of the innate and adaptive immune responses (Theoharides *et al.*, 2010).

Mast cells control immune responses by detecting antigens. Immune response activation is reliant upon IgE-dependent and IgE-independent mechanisms. IgE-independent activation is not entirely understood but likely occurs when mast cells encounter eosinophils (Munitz *et al.*, 2003). Mast cell degranulation, the process by which cytotoxic molecules are released from granules inside mast cells, is activated via the IgE-dependent mechanism (Krystel-Whittemore *et al*., 2016). The IgE-dependent detection system proceeds via mast cells binding IgE antibodies to their Fc receptors (Theoharides *et al.*, 2010). Mast cell degranulation does not occur until enough IgE antibodies, already bound to Fc receptors on the mast cell, bind to enough antigens (Theoharides *et al.*, 2010). This IgE antibody-antigen complex indirectly binds Fc receptors to the antigens in a process called Fc cross-linking (Caslin *et al.*, 2018). Fc cross-linking occurs when the Fc receptors cluster together around an antigen, signaling for degranulation (Caslin *et al.*, 2018). During degranulation, the granules contained within the mast cell are released to eliminate or flush out pathogens (Theoharides *et al.*,

2010). The Fc cross-linking phenomenon and subsequent degranulation is shown in Figure 1.1. Among other cytotoxic molecules, histamine is one of the contents released during degranulation (Caslin *et al.*, 2018). Histamine promotes inflammation by binding to histamine receptors on the surface of cells. Activation of these receptors in the gastrointestinal tract increases smooth muscle contractions, causing vomiting and diarrhea (Krystel-Whittemore *et al*., 2016).

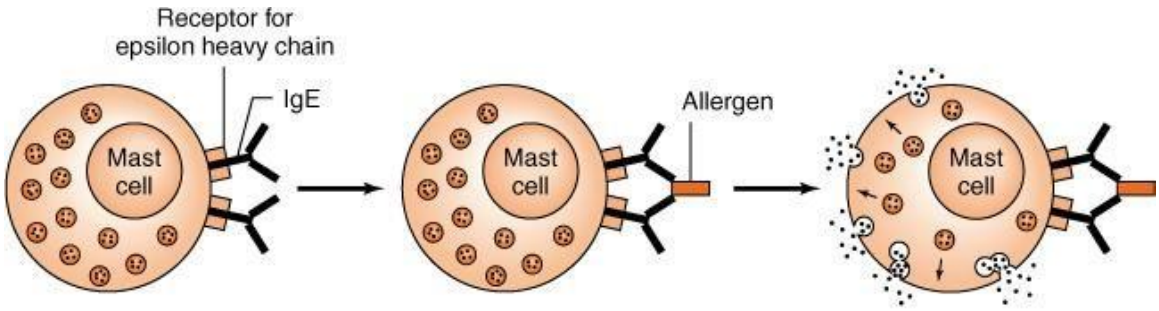

Figure 1.1: Mast Cell Degranulation. The IgE antibodies produced by plasma cells are bound by Fc receptors on mast cells. The IgE antibodies bind to antigens and signal for degranulation. Reprinted from Review of Medical Microbiology and Immunology, 11th Edition, by W. Levinson.

While not entirely understood, it is known that autophagy, the mechanism by which cytosolic substances are delivered to the lysosome for degradation, is crucial to mast cell degranulation (Nakano *et al*., 2011). Mast cell degranulation and autophagy play cooperative roles in eliminating pathogens. Autophagosomes are thought to derive from the endoplasmic reticulum (Itakura *et al.,* 2012). The autophagosome is formed when the isolation membrane elongates and encloses an area of cytoplasm. After the

autophagosome is formed, the outer membrane fuses with a lysosome causing degradation, as shown in Figure 1.2.

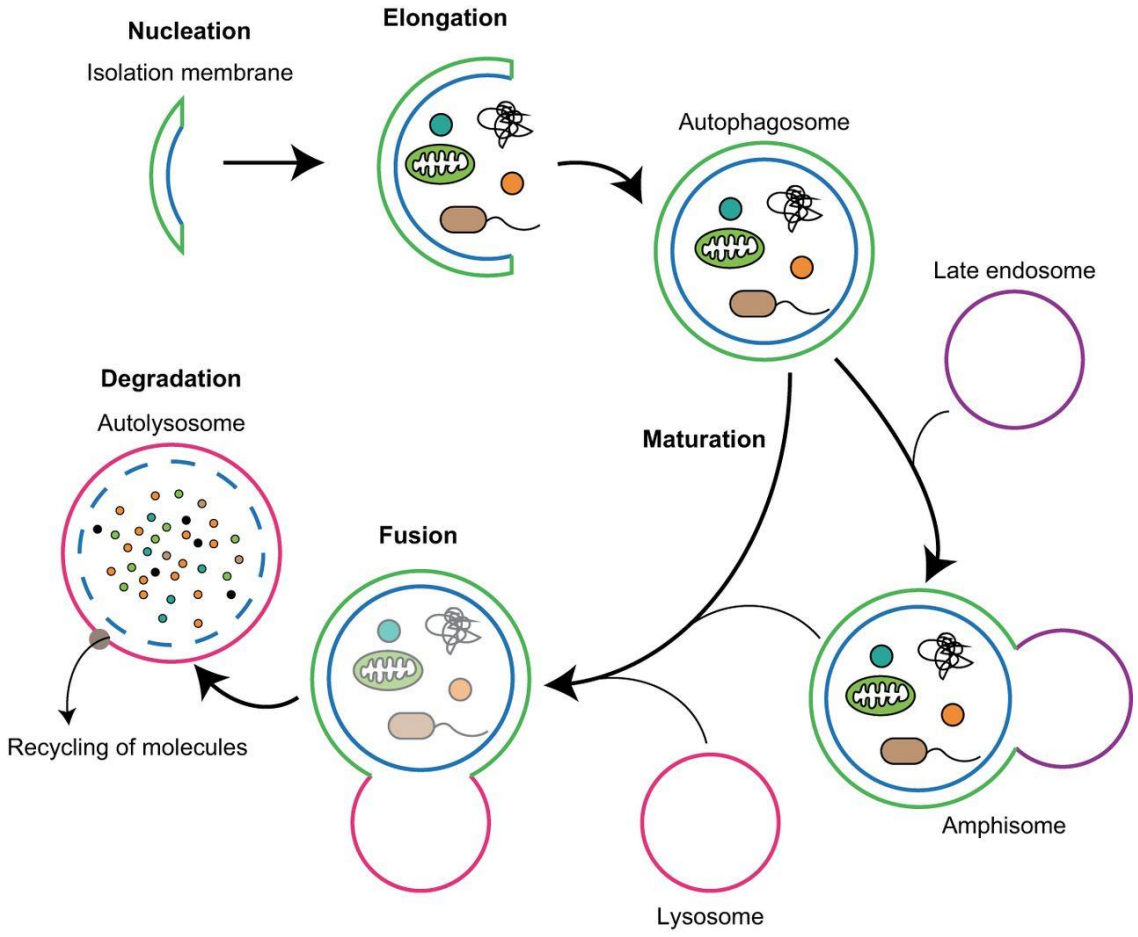

Figure 1.2: Autophagosome Formation and Lysosomal Fusion. After the isolation membrane elongates and encloses cytoplasmic contents forming the autophagosome, the autophagosome is unable to fuse with a lysosome until Stx-17 forms a complex with SNAP29 and VAMP8. Reprinted from New Insight into Autophagosome-Lysosome Fusion by Nakamura and Yoshimori, 2017.

Mast cell degranulation and autophagy require Soluble N-ethylmaleimide-sensitive factor attachment protein receptors (SNAREs), which mediate all intracellular membrane fusion along the secretory and endocytic pathways (Chen *et al.*, 2001). SNAREs are a family of proteins that mediate the transport and fusion of secretory vesicles with membranes in mast cells, resulting in degranulation, and mediate the fusion of autophagosome with lysosome, resulting in degradation of autophagosomal contents (Palfreyman & Jorgensen, 2010). SNARE proteins and the complexes these proteins form are of paramount importance to the pathogenic response in the host. Syntaxin 17 (Stx-17) was found to be an autophagosome-soluble SNARE protein that has a unique hairpin structure. This structure was found to be mediated by two transmembrane domains that both contain a glycine zipper (Itakura *et al.*, 2013). It was later revealed that Stx-17 interacts with SNAP29 and Vesicle Associated Membrane Protein 8 (VAMP8), SNAREs that are required for fusion between autophagosomes and lysosomes.

SNAREs can be divided into two categories, v-SNAREs and t-SNAREs. The v-SNAREs are present in the membranes of transport vesicles during budding and t-SNAREs are present on the target membrane (Chen *et al.*, 2001). Syntaxins and SNAPs are t-SNAREs, and VAMPs are v-SNAREs. SNARE complexes form when v-SNAREs and t-SNAREs interact, inducing a structural stress within their respective membranes. The membranous stress causes simultaneous fusion of the two membranes and formation of a pore, as seen in Figure 1.3. The exact mechanism for membrane fusion is not entirely known, and it is understood that a variety of other proteins are present (Palfreyman & Jorgensen, 2010).

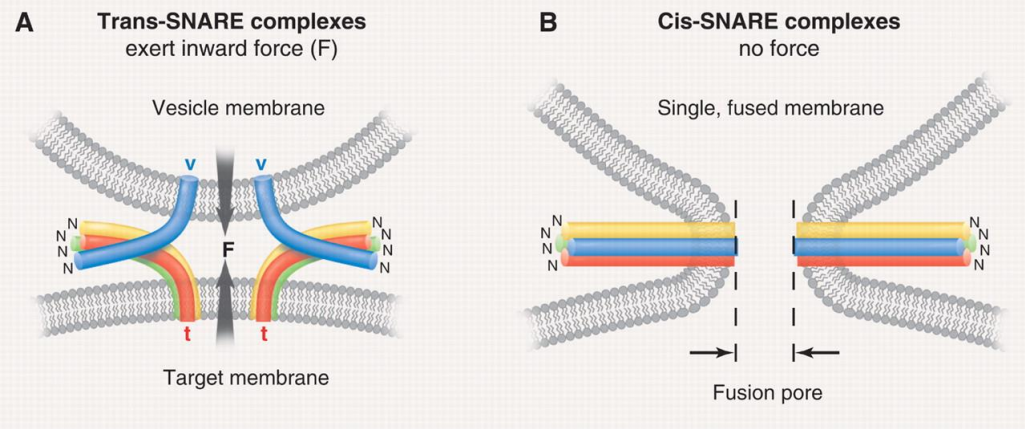

Figure 1.3: Trans and Cis-SNARE Complex and Fusion. The v-SNAREs (blue) form a trans-SNARE complex with the t-SNAREs (red, yellow, and green) on the target membranes. This trans-SNARE complex exerts inward stress on the membrane, forcing membrane fusion. After the membranes fuse, the trans-SNARE complex becomes a cis-SNARE complex with all SNAREs residing on the same membrane. Reprinted from Membrane Fusion: Grappling with SNARE and SM Proteins by T. C. Sudhof and J. E. Rothman, 2009.

Syntaxin-17, a key SNARE in membrane fusion for autophagy, was identified as a target for infection and proliferation by herpesvirus. Although localized to mitochondria-ER interfaces and mature autophagosomes, Stx-17 is ubiquitously expressed (Itakura *et al.*, 2012; Kimura *et al.*, 2018). As a ubiquitously expressed protein, Stx-17 serves as a potential target for herpesviruses. Herpes simplex virus (HSV) is sexually transmitted and infects more than 500 million people (Looker *et al*., 2008). HSV-1 and HSV-2 can establish latent infections, i.e., the virus become dormants after initial infection and is reactivated by stressors in sensory neurons, causing lesions upon reactivation (Whitley & Rolzman, 2001).

The complete infective virion is composed of four main structures, the DNA core, the protein shell or capsid, the tegument, and the surrounding envelope. There are 23 unique tegument proteins in a herpes virion and they have a multitude of functions. Some of these functions include the transactivation of viral genes, modulation of host protein expression, and alteration of the immune response (Henaff *et al.*, 2013). Tegument proteins have also been observed to interact with viral glycoproteins and collect near the trans-Golgi network, suggesting that the Golgi is the final packaging site of the virion before release (Henaff *et al.*, 2013). Research has discovered herpesvirus tegument proteins interacting with the SNARE complex needed to initiate autophagy (Kelly *et al.,* 2009). A recent study was performed on tegument proteins ORF33 and ORF38 in Kaposi Sarcoma Herpes Virus (KSHV). These proteins are conserved in all herpesviruses and were discovered to be required for optimal production of infectious virions (Wu *et al.,* 2016).

Recent studies have indicated that mast cells could potentially be a long-term reservoir for herpesviruses, as seen in the study of KSHV (Ayers *et al.,* 2018). Research into KSHV infected mast cells found evidence that suggests mast cells are permissive to KSHV infection and gene expression (Ayers *et al.,* 2018). Mast cells were observed for over two weeks post infection and viral gene expression was noted to be elevated for the entire duration, further suggesting mast cells as a reservoir for herpesvirus (Ayers *et al.,* 2018).

Another study found Enterovirus (EV) D68 disrupts autophagic degradation via inhibition of SNARE complex formation during autophagosome-lysosome fusion. The proteins forming the SNARE complex responsible for fusion, Stx-17, SNAP29, and

VAMP8, were monitored after infection of H1HeLa cells with EV-D68. The autophagosome associated SNAREs Stx-17 and SNAP29 were observed. An increase in Stx-17 and decrease in SNAP29 were noted. SNAP29 reduction was attributed to cleavage by protease EV-D68 3C (Corona *et al.*, 2018). It was determined that EV-D68 promoted autophagic signaling but prevented degradative steps of autophagy (Corona *et al.*, 2018). The decrease in SNAP29 allowed for viral replication, but not degradation.

As mentioned above, Stx-17 plays a key role in fusion of the autophagosome with the lysosome. Thus, Stx-17 is a key SNARE in the maintenance of homeostasis. Further study of the targeting of this SNARE and the complex it forms with SNAP29 and VAMP8 during autophagosome-lysosome fusion by tegument proteins is critical for understanding and preventing herpesvirus infections. Studying Stx-17 will not only provide insight into the mechanism by which membrane fusion occurs, but also offer invaluable data for the targeting mechanism of conserved tegument proteins in herpesviruses. To perform future research on the interaction between the Stx-17 SNARE and viral tegument proteins, a readily available supply of the Stx-17 protein is necessary. It is the goal of this study to insert the Stx-17 gene into a plasmid vector and introduce the chimeric plasmid into a strain of *E. coli* for protein synthesis.

Chapter 2: Materials and Methods

2.1 Study Overview and Plasmid Information

The syntaxin 17 (*Homo sapiens*) clone was ordered from addgene. The cDNA for the Stx-17 insert is stored within the *E. coli* strain HXB_0J82. The strain was ordered from addgene, catalog number 89942, and the total recombinant plasmid is 7140 bp in length. The Stx-17 insert is 909 bp. The gene sequence was shipped in a recombinant plasmid, Figure 2.1, that is unsuitable for gene expression. The Stx-17 gene sequence was excised from the recombinant plasmid and ligated to a vector containing a sequence for an isolation protein. The vector pMBP-parallel1 was chosen due to the presence of the gene for maltose binding protein, MBP, and restriction sites corresponding with enzymes present in lab. The cDNA for the pMBP-parallel1 vector is stored in the strain HXB_OC21. The cell origin is C2992, a DH5α derivative. The vector sequence is 6,724 bp in length, as seen in Figure 2.2. Maltose binding protein allows for Stx-17 to be expressed with a marker that can be used to collect the expressed protein during an affinity column purification.

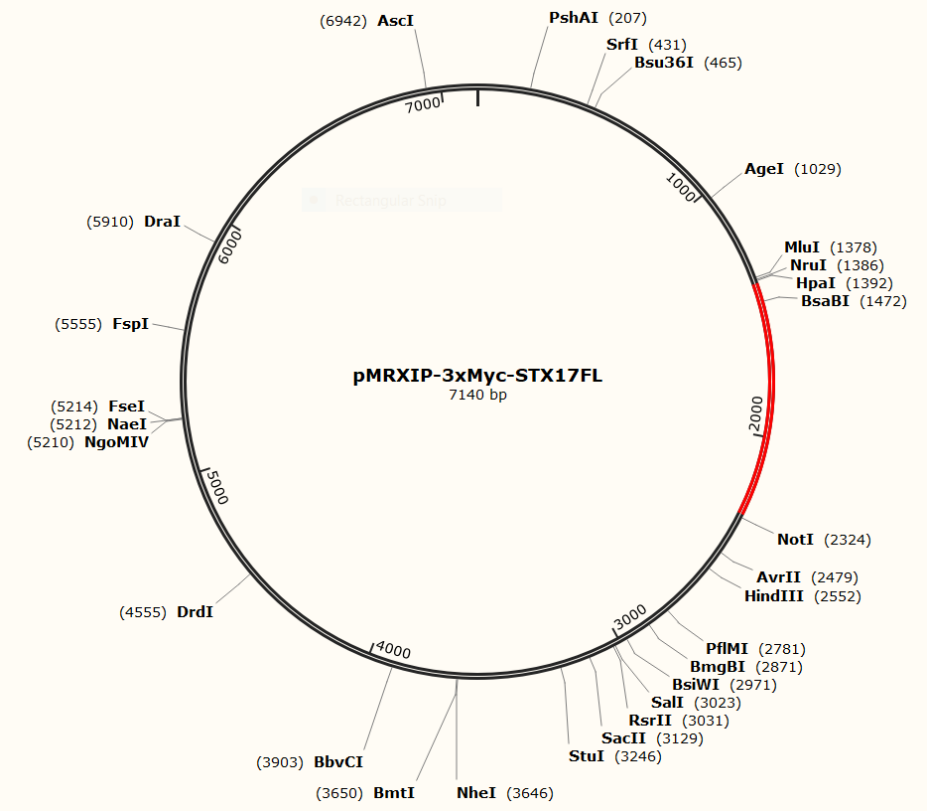

Figure 2.1: pMRXIP-3xMyc-STX17FL Map. The map is of the recombinant plasmid from addgene prior to plasmid isolation. The portion of the map outlined in red contains the insert sequence for Stx-17 prior to excision.

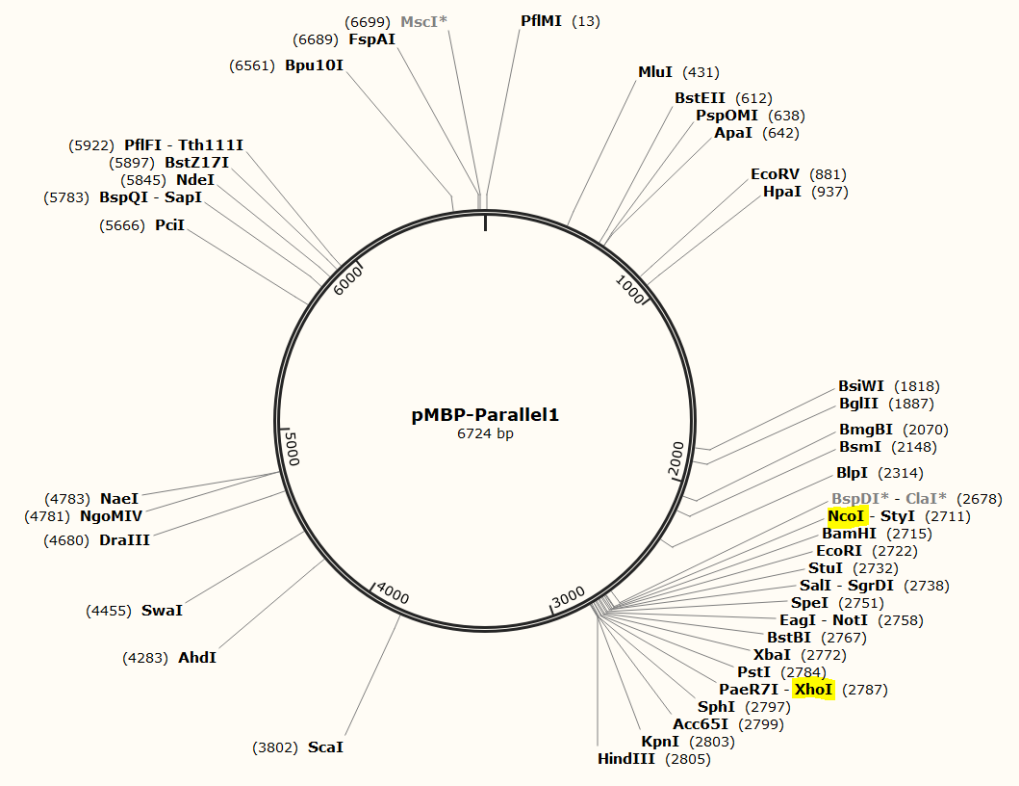

Figure 2.2: pMBP-parallel1 Restriction Enzyme Map. NcoI and XhoI were selected as the restriction sites for vector pMBP-parallel1.

2.2 Vector Plasmid Inoculation

After obtaining the appropriate vector stock from the freezer, a sterile loop was applied to the top of the stock. This sterile loop was then spread over an AMP 100 agar plate allowing room between streaks to allow for the potential isolation of a single colony. The plate was placed into the 37 ºC incubator overnight.

A single colony was selected and inoculated into a tube with 5 ml of LB and 5 µl of ampicillin. The tube was left in the 37 ºC shaking incubator overnight.

2.3 Insert Plasmid Inoculation

The appropriate Stx-17 stock was obtained from the freezer a sterile loop was applied to the top of the stock. This sterile loop was then spread over an AMP 100 agar plate as to allow room between streaks and allow for the potential isolation of a single colony. The plate was placed into the 37 ºC incubator overnight.

A single colony was selected and inoculated into a tube with 5 ml of LB and 5 µl of ampicillin. The tube was left in the 37 ºC shaking incubator overnight.

2.4 Vector and Insert Plasmid Isolation

The cultures were isolated using the QIAquick Plasmid Isolation kit, category no. 27106. The tube was placed on ice and 1.5 ml was extracted from the tube and placed into a 2.0 ml tube and centrifuged at 13k rpm for 1 minute at room temperature. The supernatant was decanted, another 1.5 ml was added, and the tube was centrifuged again. The supernatant was decanted and the remaining 2 ml from the culture was stored.

The tubes were vortexed to resuspend the pellet. Each tube received 250 µl of Buffer P1 to aid resuspension. 250 µl of Buffer P2 was added to release the plasmid DNA from the bacterial cells. The tubes were then inverted 10 times and placed at room temperature for five minutes. 350 µl of N3 Buffer was added to neutralize the solution. The tubes were placed on ice for 10 minutes and subsequently inverted 10 times.

The tubes were centrifuged at 13k rpm for 10 minutes at 4 ºC. The supernatant was transferred to three columns and centrifuged at 13k rpm for 1 minute at room temperature. The flow through was discarded and 500 µl of Buffer PB was added to the column to aid DNA binding to the column. Tubes were subsequently centrifuged at 13k rpm for 1 minute at room temperature to remove any residual buffer. Flow through was

discarded and 750 µl of Buffer PE was added to the columns to remove excess salts from the column membrane. The tubes were centrifuged at 13k rpm for 1 minute at room temperature and the flow through was discarded. The columns were placed back into their respective tubes and centrifuged twice more to remove residual buffer. The columns were transferred to sterile 1.5 ml tubes and allowed to sit for 5 minutes. 50 µl of sterilized Millipore water was added to the membranes of the columns and allowed to sit for 1 minute to elute the bound DNA. The columns were centrifuged at 13k rpm for 1 minute at room temperature. The flow through was collected and placed back onto the respective column membranes and centrifuged once more. The flow through was collected and analyzed via nanodrop machine.

2.5 PCR Amplification of Stx-17 and Gel Electrophoresis Confirmation

Aliquots of 10x pfu buffer, 10 µM forward primer, 10 µM reverse primer, 10 µM dNTPs, insert template, and pfu DNA polymerase were obtained from the -20 ºC freezer and allowed to slowly thaw on ice before short centrifugation. While on ice, 40 µl of sterilized Millipore water, 5 µl of 10x pfu buffer, 1 µl of the 10 µM forward primer, 1 µl of 10 µM reverse primer, 1 µl of the 10 µM dNTPs, 1 µl of the insert template, and 1 µl of pfu DNA polymerase were added to a PCR tube. The tube was centrifuged briefly to mix and placed into the thermocycler.

The protocol for the thermocycler was a 5-minute hold at 94ºC followed by two main cycles. The first cycle was repeated 5 times and consisted of 94 ºC for 30 seconds, 55 ºC for 30 seconds, and 72 ºC for 55 seconds. The second cycle was run 25 times and consisted of 94 ºC for 30 seconds, 60 ºC for 30 seconds, and 72 ºC for 55 seconds. Then

the thermocycler was set to 72 ºC for 10 minutes followed by a 4 ºC hold until removal from the thermocycler.

A gel electrophoresis was performed to confirm the size of the insert PCR product. The 0.7% gel was created via combining 0.35 g of agarose with 50 ml of 0.5X TBE buffer and boiled for 45 seconds, mixed by swirling, boiled for 15 seconds, mixed by swirling, boiled for another 15 seconds, followed by mixing by swirling. The mixture was allowed to cool for 10 minutes before pouring into a casting tray with comb. After solidifying for 45 minutes, the gel was placed into a running tray and the tank was filled to the max line with 0.5X TBE buffer, covering the gel. On a sterile piece of parafilm, 1 µl of 100 bp DNA ladder, 2 µl of GelRed, and 7 µl of sterilized Millipore water were gently mixed by pipetting and placed into the first well of the gel. The second well contained 5 µl of the unpurified PCR product, 2 µl of GelRed, and 3 µl of sterilized Millipore water. The gel was run for 75 minutes at 80 V and analyzed.

2.6 PCR Purification

The QIAquick PCR Purification Kit, category no. 28104, was used to purify the insert PCR product. To enable efficient binding of the DNA to the spin column membrane, 5 volumes (225.0 µl) of PB Buffer were added to 45.0 µl of the PCR product and mixed by brief centrifugation. A column was placed into a 2 ml collection tube and the sample mixture was added to the column. To bind the DNA to the membrane, the column was spun at 13k rpm at room temperature for 1 minute. The flow through was discarded and the column was placed back into the collection tube. To remove excess salts from the membrane, 750 µl of Buffer PE was added to the column and centrifuged at 13k rpm at room temperature for 1 minute. The flow through was discarded and the

column was placed back in the collection tube. Centrifugation and disposal steps were repeated twice more. The column was placed into a clean 1.5 ml collection tube. DNA was eluted by adding 30 µl of sterilized Millipore water directly to the membrane and let stand for 1 minute. The column was spun for 1 minute at 13k rpm at room temperature. The flow through was collected and placed directly on the membrane again and let stand for 1 minute. The column was spun again for 1 minute at 13k rpm at room temperature. The flow through was analyzed on the nanodrop machine.

2.7 Double Digest of Vector and Insert

The purified Stx-17 PCR product, isolated pMBP-parallel1, 10X NEB Cutsmart Buffer, XhoI, and NcoI were removed from the freezer and allowed to thaw on ice. After thawing, they were centrifuged and placed back on ice. Two tubes were prepared and labeled, Tube A for the Stx-17 insert and Tube B for the pMBP-parallel1 vector. In Tube A, 33 µl of sterilized Millipore water, 10 µl of the purified Stx-17 PCR product, 5 µl of the 10X NEB Cutsmart Buffer, 1 µl of the NcoI enzyme, and 1 µl of the XhoI enzyme were added. In Tube B, 32.5 µl of sterilized Millipore water, 10.5 µl of isolated pMBP-parallel1, 5 µl of 10X NEB Cutsmart Buffer, 1 µl of NcoI enzyme, and 1 µl of the XhoI enzyme were added. Both tubes were briefly centrifuged to make sure they were evenly mixed and placed into a 37 ºC water bath for 3 hours. After the water bath, both tubes were briefly centrifuged again and placed on a 65 ºC heat block for 20 minutes to neutralize digestion by the enzymes. Tube A, the insert, was placed into the -20 ºC freezer. Tube B, the vector, was treated with 1 µl of rSAP to remove any unincorporated dNTPs, briefly centrifuged, and placed in a 37 ºC water bath for 1 hour. Afterwards, Tube

B was centrifuged and placed on a 65 ºC heat block for 20 minutes to neutralize the rSAP. Tube B was placed into the -20 ºC freezer with Tube A.

2.8 Gel Electrophoresis Confirmation

A gel electrophoresis was performed to confirm the size of the digested products and to prepare them for gel extraction purification. As before, the 0.7% gel was created by combining 0.35 g of agarose with 50 ml of 0.5X TBE buffer and boiled for 45 seconds, mixed by swirling, boiled for 15 seconds, mixed by swirling, boiled for another 15 seconds, followed by mixing by swirling. The mixture was cooled for 10 minutes at room temperature before pouring into a casting tray with comb. Unlike before, wells 2 and 3 along with 5 and 6 were combined to allow for all of the digested contents of Tubes A and B to be placed in one well. After solidifying for 45 minutes, the gel was placed into a running tray and the tank was filled to the max line with 0.5X TBE buffer, covering the gel. On a sterile piece of parafilm, 1 µl of 1kb DNA ladder, 2 µl of GelRed, and 7 µl of sterilized Millipore water were gently mixed by pipetting and placed into the first well of the gel. The combined well of lanes 2 and 3 contained all of the contents of Tube A mixed with 2 µl of GelRed. Well 4 was left empty to allow enough separation between the large combined wells. Combined wells 5 and 6 contained all of the digested contents of Tube B along with 2 µl of GelRed. The gel was run for 75 minutes at 80 V and analyzed under the orange UV box to prevent damage to the DNA bands.

2.9 Vector and Insert Gel Extraction

Using a clean scalpel for each band, the digested Stx-17 insert band and pMBP-parallel1 vector band were excised from the gel and each placed into a clean 2 ml tube of known weight. Each tube was weighed again to find the weight of the gel and band

contained in each respective tube. Using the QIAquick Gel Extraction Kit, category no. 28704, band extraction began.

Both bands weighed 130 mg, so 390 µl of Buffer QG (3 volumes) were added to each tube to solubilize the agarose gel that the band is contained in. Each tube was placed on a 55 ºC heat block and vortexed every 2 minutes for 10 minutes. The mixture was monitored to remain yellow. To precipitate the DNA from solution, 130 µl of isopropanol (1 volume) was added. A column was placed into a 2 ml collection tube and the mixture was added. The column was centrifuged at 13k rpm for 1 minute at room temperature and the flow through was discarded. 500 µl of Buffer QG was added and the column was centrifuged at 13k rpm for 1 minute at room temperature. The flow through was discarded and 750 µl of Buffer PE was added. The column was left to stand for 5 minutes and subsequently centrifuged at 13k rpm for 1 minute at room temperature. Flow through was discarded and the column was spun one more time. The column was placed into a clean 1.5 ml collection tube and 30 µl of sterilized Millipore water was added directly to the membrane of the column. The column was left to stand for 5 minutes and then centrifuged at 13k rpm for 1 minute at room temperature. The flow through was collected and analyzed via nanodrop.

2.10 Vector and Insert Ligation

It was determined that a 4:1 ratio would be needed and the vector, pMBP-parallel1, would be required in a 50 ng amount. It was determine via equation, $4 \times (\text{insert bp}/\text{vector bp})(50 \text{ ng}) = \text{insert ng}$, that the insert would be required in a 27.04 ng amount. The nanodrop values collected for the vector and insert after gel extraction were used to determine that 2.8 µl of vector pMBP-parallel1 and 2.0 µl of insert Stx-17

would be required for the ligation reaction. The NEB T4 DNA Ligase, 10X T4 DNA Ligase Buffer, and gel purified products were removed from the -20 ºC freezer and allowed to thaw on ice and briefly centrifuged.

Two tubes were prepared on ice, Tube A for the actual ligation of insert and vector while Tube B contained the vector only as a negative control. In Tube A, 2.7 µl of sterilized Millipore water, 2.0 µl of Stx-17, 2.8 µl of pMBP-parallel1, 1.5 µl of 10X T4 DNA Ligase Buffer, and 1 µl of T4 DNA Ligase were added. In Tube B, 4.7 µl of sterilized Millipore water, 2.8 µl of pMBP-parallel1, 1.5 µl of 10X T4 DNA Ligase Buffer, and 1.0 µl of T4 DNA Ligase were added.

The tubes were both briefly centrifuged to thoroughly mix the contents of each. Both tubes were then placed in a thermocycler for 17 hours at 17 ºC.

2.11 Recombinant Plasmid Transformation into NovaBlue Cells

Aliquots of NovaBlue cells from the -80 ºC freezer along with ligation products A and B from the -20 ºC freezer were removed and allowed to thaw on ice. All tubes were gently mixed after thawing by flicking. In separate tubes, 2 µl of ligation products A and B were mixed with 20 µl of NovaBlue competent cells. As a positive control to ensure the NovaBlue cells were viable, a third tube was prepared with 20 µl of competent cells and 1 µl of PUC. All tubes were mixed via gentle flicking and placed on ice for 45 minutes. All tubes were placed on a heat block for 45 seconds at 42 ºC. All tubes were then placed on ice for 30 minutes. To aid in molecular uptake and recovery of the competent cells, 100 µl of SOC media was added to tubes A and B. The third positive control tube received a mixture of 110 µl of LB and 10 µl SOC media. To further aid in recovery, all tubes were placed in 37 ºC incubator for 1 hour. During incubation, 3 AMP

100 agar plates were prepared and labelled. After recovery, the tubes were centrifuged for 5 minutes at 4k rpm at room temperature. Most of the supernatant was removed and the pellets were resuspended. Each sample was plated onto their respective plates. All plates received 7 glass beads and were rotated vigorously for 15 seconds to thoroughly spread the sample through the plate. Plates were placed in the 37 ºC incubator for 20 minutes facing up so the samples were able to absorb into the agar. After the 20 minutes, the plates were flipped over and left overnight in the 37 ºC incubator.

2.12 Potential Transformation Isolation

Three separate colonies on the transformation plate were inoculated into three test tubes containing 5 ml of LB and 5 µl of AMP 100. These three tubes were placed into the 37 ºC shaker incubator overnight. The tubes were removed and placed on ice. 1.5 ml was extracted from each tube and placed into a corresponding 2.0 ml tube and centrifuged at 13k rpm for 1 minute. The supernatant was decanted and another 1.5 ml was added to the respective tube and centrifuged. The supernatant was decanted, and the remaining 2 ml was stored.

Using QIAquick Plasmid Isolation Kit, category no. 27106, chimeric plasmid isolation continued. Each tube was vortexed until the pellet disappeared and received 250 µl of Buffer P1 to resuspend. This was followed by the addition of 250 µl of Buffer P2, a lysis buffer that allows for the release of plasmid DNA from bacterial cells. The tubes were then inverted 10 times and placed at room temperature for five minutes. Next 350 µl of N3 Buffer was added to neutralize the solution. The tubes were placed on ice for 10 minutes and subsequently inverted 10 times.

The tubes were centrifuged at 13k rpm for 10 minutes at 4 ºC. The supernatant was transferred to three columns and centrifuged at 13k rpm for 1 minute at room temperature. The flow through was discarded and 500 µl of Buffer PB was added to the column to aid DNA binding to the column. Tubes were subsequently centrifuged at 13k rpm for 1 minute at room temperature to remove any residual buffer. Flow through was discarded and 750 µl of Buffer PE was added to the columns to remove excess salts from the column membrane. The tubes were centrifuged at 13k rpm for 1 minute at room temperature and flow through was discarded. The columns were placed back into their respective tubes and centrifuged twice to remove any residual buffer. The columns were transferred to 1.5 ml tubes and allowed to sit for 5 minutes. To elute the DNA, 50 µl of sterilized Millipore water was added to the membranes of the columns and allowed to sit for 1 minute. The columns were centrifuged at 13k rpm for 1 minute at room temperature. The flow through was collected and placed back onto the respective column membranes and centrifuged once more. The flow through was collected and analyzed by nanodrop.

2.13 Double Digestion and Gel Electrophoresis Confirmation

Colony Forming Unit (CFU) B, the sample with the highest concentration at 68.1 ng/µl, was selected for double digestion and gel electrophoresis. On ice, 28 µl of sterilized Millipore water, 15 µl of the potential plasmid, 5 µl of the 10X NEB Cutsmart Buffer, NcoI enzyme, and XhoI enzyme were added to a tube. The tube was placed in a 37 ºC water bath for 1 hour. Subsequently, the tube was briefly centrifuged and placed on a 65 ºC heat block for 20 minutes to neutralize the enzymes.

Two gel electrophoresis were performed on the sample. The first gel was 0.7% and contained 1 µl of 1kb ladder, 2 µl of GelRed, and 7 µl of sterilized Millipore water in the first well. The second well contained 6 µl of the double digested CFU B, 2 µl of GelRed, and 2 µl of sterilized Millipore water. The gel was run for 75 minutes at 80V and analyzed under the imager.

On the second 0.7% gel, the double digested and undigested CFU B samples were run. Well 1 contained 1 µl of 1kb ladder, 2 µl of GelRed, and 7 µl of sterilized Millipore water. The second well contained 4 µl of sterilized Millipore water, 4 µl of undigested recombinant plasmid, and 2 µl of GelRed. The third well contained 4 µl sterilized Millipore water, 4 µl of double digested recombinant plasmid, and 2 µl of GelRed. The gel was subsequently imaged and analyzed.

Chapter 3: Results

To investigate the biochemical and functional interaction between the tegument proteins and Stx-17, a readily available supply of the SNARE protein is necessary. The experiments performed explored the concept of Stx-17 cDNA transformation into *E. coli* strain suitable for protein production. Due to the presence of maltose binding protein and prior experience in the research lab, pMBP-parallel was chosen as the vector. Samples of the vector and insert were plated on their respective plates and colonies were allowed to grow overnight. The next day colony forming units, CFUs, were isolated and the concentrations were analyzed via nanodrop machine. The nanodrop measures absorbance and calculates the concentration of nucleic acids. The pMBP-parallel1 sample was determined to have a 94.1 ng/µl concentration and had A260/280 and A260/230 of 1.84

and 2.12, respectively (Table 3.1). The Stx-17 sample was determined to have a 385.1 ng/µl concentration and had A260/280 and A260/230 of 1.87 and 2.31, respectively (Table 3.1). These values indicate the sample contained a high concentration of vector and the sample was pure. This was determined because a A260/280 value around 1.8 indicates a pure DNA sample without proteins contamination. Also, a A260/230 value around 2 indicates the presence of no unwanted organic compounds.

Table 3.1: Concentration of Vector and Insert Plasmid Isolation.

Plasmid/Strain	Concentration: ng/µl	A260/280	A260/230
pMBP-parallel1/HXB_OC21	94.1	1.84	2.12
Stx-17/HXB_OJ82	385.1	1.87	2.31

To obtain a workable amount of Stx-17 cDNA, PCR was performed on the insert sample. Gel electrophoresis was performed prior to PCR purification to verify the band size of the insert. The unpurified insert PCR product was run on a 0.7% gel with a 100 bp ladder to determine the size of the sample (Fig 3.1). The unpurified insert PCR product band appeared between the 1000 bp and 1150 bp bands despite the insert being 909 bp in length.

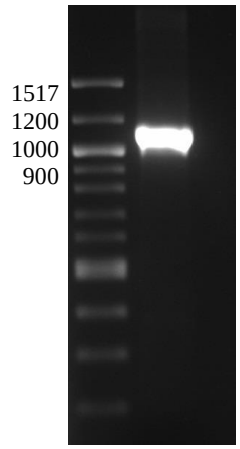

Figure 3.1: Unpurified Stx-17 PCR Gel Electrophoresis.

Lane 1 contained 1 µl of 100 bp DNA ladder, 2 µl of GelRed, and 7 µl of sterilized Millipore water. Lane 2 contained 5 µl of the unpurified PCR product, 2 µl of GelRed, and 3 µl of sterilized Millipore water.

Although the insert band appeared to have more base pairs on the gel electrophoresis than anticipated, the sample was purified, and the concentration was analyzed via nanodrop. The sample was determined to have a 99.1 ng/µl concentration and had A260/280 and A260/230 values of 1.8 and 2.5, respectively (Table 3.2). Although 2.5 is slightly higher that the desired number, it was not high enough to indicate any serious contamination.

Table 3.2: Concentration of Purified Insert Stx-17 PCR Product.

Sample	Concentration: ng/µl	A260/280	A260/230
Stx-17	99.1	1.8	2.5

The vector and purified insert samples were prepared for ligation by double digestion and purified by gel extraction. Double digestion created sticky ends, conducive

for ligation. Following digestion, the samples were run alongside a 1kb ladder to confirm their sizes and to extract the corresponding bands (Fig 3.2). Double digested Stx-17 presented between 1,000 and 1500 bp. The double digested pMBP-parallel1 began around the 6,000 bp band.

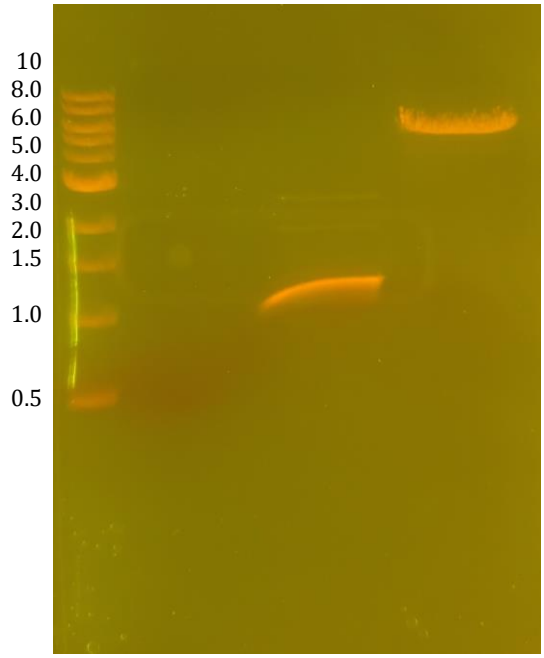

Figure 3.2: Double Digested Insert and Vector Prior to Gel Extraction. Lane 1 contained 1 µl of 1kb ladder, 2 µl of GelRed, and 7 µl of sterilized Millipore water. Combined lanes 4 and 5 contained 2 µl of GelRed and 50 µl of the double digested insert, Stx-17. Combined lanes 6 and 7 contained 2 µl of GelRed and 50 µl of the double digested vector, pMBP-parallel1.

Once the excised bands underwent gel extraction, their nanodrop concentrations were taken. The concentrations of the double digested insert and vector were not retained after the gel extraction indicated by Table 3.3. An insert value of 19.7 and vector value of 17.9 indicated low concentrations and absorption values of 1.9 and 0.1 for both samples indicate impurities. The double digested samples were not concentrated, nor were they pure.

Table 3.3: Concentration of Gel Extracted Insert and Vector After Restriction Double Digestion.

Plasmid Sample	Concentration: ng/µl	A260/280	A260/230
Stx-17	19.7	1.9	0.1
pMBP-parallel1	17.9	1.9	0.1

Despite receiving low gel extraction values, the samples underwent a gel electrophoresis to confirm their size prior to ligation. The results from the ladder are inconclusive as the ladder did not run properly; however, Stx-17 appears to be concentrated around the 1,500 bp band and pMBP-parallel1 begins slightly before the 8,000 bp band and ends around the 10,000 bp band (Fig 3.3). These values indicate the samples are heavier than they should be.

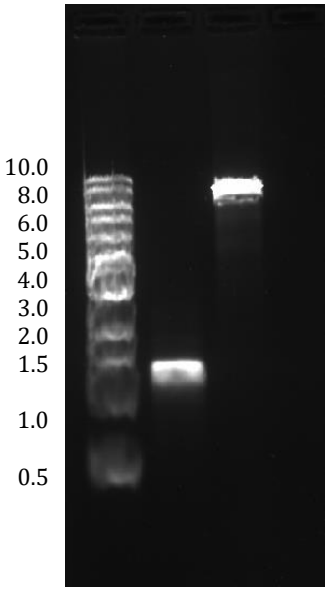

Figure 3.3: Gel Purified and Double Digested Insert and Vector.

Lane 1 contains a 1 kb ladder that did not separate properly during the run. Lanes 2 and 3 contained the 4 µl of gel extracted Stx-17 and pMBP-parallel1 samples, 2 µl GelRed, and 4 µl of sterilized Millipore water, respectively.

Although the concentration values and gel bands did not confirm what was expected, the insert and vector were ligated, transformed, and plated twice. Three plates were created for each attempt. Plate #1 was the insert and vector, #2 was the vector only, and #3 was the competent cells only. Plate #2 was to ensure vector did not fold and ligate back onto itself, this plate was not supposed to have any growth. Plate #3, contained only the NovaBlue competent cells, was to confirm the viability of the cell line and to serve as a positive control. The first attempt did not see any CFUs grow on #1 or #2 (Fig 3.4). The second attempt saw 27 CFUs grow on Plate #1 after two days in the incubator (Fig 3.5). The second attempt Plate #2 did not see any CFUs (Fig 3.5).

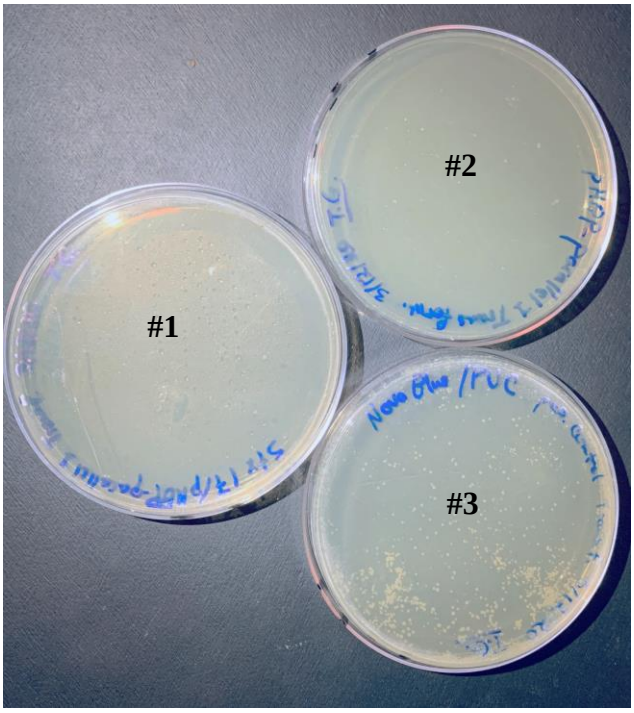

Figure 3.4: Transformation Plates for Stx-17/pMBP-parallel1 (Plate #1), pMBP-parllel1 only (Plate #2), and NovaBlue competent cells only (Plate #3).

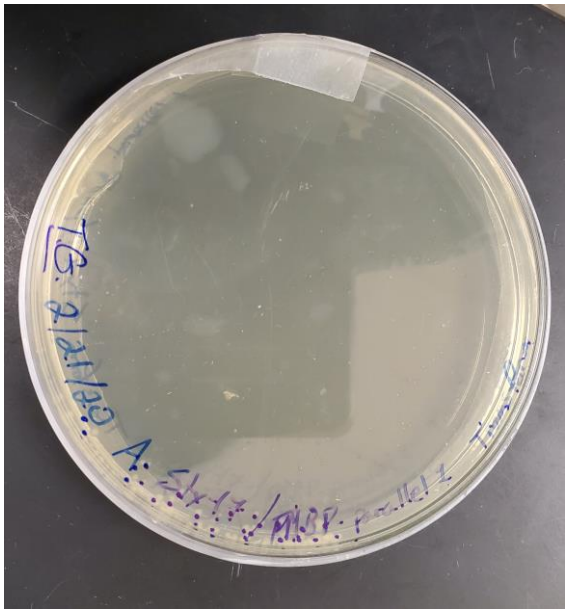

Figure 3.5: Second Transformation Plate for Stx-17/pMBP-parallel1.

Three colonies were isolated from the insert and vector plate and underwent plasmid isolation. CFU A and CFU C did not have a high concentration of plasmid, nor were their A260/230 values close to the recommended 2-2.2 threshold, indicating the samples were not pure either (Table 3.4). CFU B had a much higher concentration than the other isolated CFUs. Although none of the isolated CFUs showed pure absorption values, CFU B displayed a concentration high enough to perform double digestion and gel electrophoresis.

Table 3.4: Concentration of Three Isolated CFUs from Transformation Plate.

Potential Transformation Sample	Concentration: ng/µl	A260/280	A260/230
Potential CFU A	13.6	1.53	0.55
Potential CFU B	68.1	1.54	0.58
Potential CFU C	5.3	1.54	0.53

Potential CFU B was double digested and two gels were run for band size confirmation in Fig 3.6 and Fig 3.7. The first gel (Fig 3.6) indicated faint bands at approximately the desired size. The first band at 1,000 bp corresponds with the size of Stx-17 at 909 bp. The second band at around the 8,000 bp mark could correspond to pMBP-parallel1 at 6,724 bp. The band at the top of the gel may correspond to relaxed DNA that was not properly digested and did not travel far.

The sample was run a second time to confirm the size seen in Fig 3.7. Both digested and undigested were run; however, no bands appeared. As seen in Fig 3.7, lane 1 contains the 1 kb ladder and lanes 2 and 3 contain the undigested and double digested CFU B, respectively. No bands are visible in lanes 2 or 3. Lane 2 should have seen a band around 8,000 bp for the undigested plasmid. Lane 3 should have seen bands at 1,000 bp for the insert and 7,000 bp for the vector.

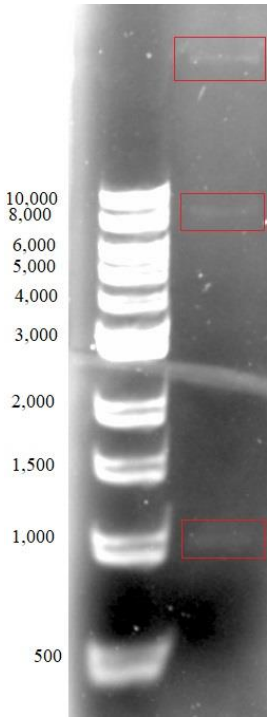

Figure 3.6: Possible Stx-17/pMBP-parallel1 Transformation Double Digest Gel Electrophoresis.

Red boxes indicate a potential band at 1,000 bp corresponds with the size of Stx-17 at 909 bp, a second band at around the 8,000 bp mark could correspond to pMBP-parallel1 at 6,724 bp, and a band at the top of the gel may indicate relaxed DNA that was not properly digested and did not travel far.

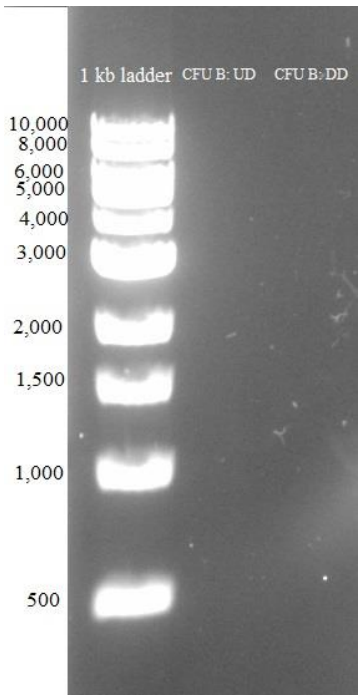

Figure 3.7: Isolated CFU B Undigested and Double Digested.

No bands were observed for the undigested and double digested CFU B.

Chapter 4: Discussion

Results from the double digested transformation plate isolate yielded inconclusive results. The second transformation attempt saw only 27 CFUs around the edge of the plate after two days in the incubator. This could indicate slow growing colonies or that the AMP100 selection was no longer effective by the second day allowing false colonies to grow on the plate. Because the colonies grew on the edge of the plate, this could indicate that the glass beads were not properly rolled as to spread the sample across the whole plate or that contamination reached the edge of the plate. Despite potentially being false colonies, 3 CFUs were isolated towards the center of the plate and purified. In spite of low A260/230 values, the CFU with the highest concentration was double digested.

The double digested potential recombinant plasmid on Fig 3.6 corresponds roughly to what was expected; however, the vector band should have been between 6kb

and 8kb instead of between 8kb and 10kb because pMBP-parallel1 is 6,724 bp. When reviewing the results with what was expected, it was not unheard of for the band to appear slightly heavier than it should be. Despite this, the results obtained from this isolation are inconclusive. The faint band at 1kb corresponds with Stx-17 insert that has 909 bp. The band at the top of the gel could potentially be undigested DNA that was nicked during the digestion and may have relaxed some of the super coiling, causing the band to not travel as far. When the gel was run again with the undigested and double digested plasmid, no bands appeared (Fig 3.7). This is interesting because the concentration of the isolated CFU B was only slightly reduced to conserve sample for potential sequencing.

It is likely that CFU B was a false positive or did not have a high enough concentration to be valuable. Currently, the data is inconclusive as to whether the ligation and transformation were successful. If the correct bands had been identified for the isolated transformation colony, the sample would need to be sent off for sequencing. Once the sequence had been obtained, it would need to be analyzed alongside the confirmed nucleotide sequence on the NCBI website. If any mutations had been detected, transformations would need to be performed again to obtain a more faithful DNA sample. After confirmation, protein isolation from the cell cultures would follow.

Addressing the low concentration for the gel extraction, the double digested vector and insert the bands were not exposed to UV light so the samples should not have been mutated or damaged. Despite this, high concentrations for the insert and vector could not be obtained. The gel extraction was performed three times. The gel slices were cut as close to the bands as possible, the gel fragments were allowed to completely

dissolve, and the 30 µl of sterilized Millipore water were applied directly to the membrane of the column and allowed to stand for 5 minutes before centrifugation.

When looking into gel extraction, low concentrations are common with the QIAquick Gel Extraction Kit, category no. 28704. Two potential issues discovered are if the spin columns are not properly sealed in their bags the membranes have the potential to dry out and if too high of a concentration is being applied to the column, the membrane will not be able to absorb all of the sample. Two possible solutions include obtaining new columns and sealing their bag between use or using multiple columns for high concentration.

Stx-17 plays a pivotal role in membrane fusion and is a target for herpesvirus tegument proteins. It is crucial to obtain a deeper understanding of this protein and to develop a method for producing it so it can be studied. Future research into this protein should pick up prior to the Gel Extraction step and focus on obtaining higher concentrations and more pure samples. When these samples are obtained, successful ligation and transformation will be more likely. After a successful transformation and isolation, sequencing will need to be performed to confirm the sample. Then protein affinity isolation can be used to purify the protein using the MBP tag. Confirmation of the protein production will allow for the method to be scaled up to produce an abundant supply of Stx-17 protein. Once a successful method of supplying Stx-17 is established, further research on herpesvirus interaction with this SNARE can be performed.

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