CHARACTERIZATION OF A PUTATIVE BNR REPEAT DOMAIN PROTEIN IN THE VIRULENCE OF *BACILLUS ANTHRACIS*

by

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Submitted in partial fulfillment of the

requirements for Departmental Honors in

the Department of Biology

Texas Christian University

Fort Worth, Texas

May 2, 2022

CHARACTERIZATION OF A PUTATIVE BNR REPEAT DOMAIN PROTEIN IN THE VIRULENCE OF *BACILLUS ANTHRACIS*

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ABSTRACT

Bacillus anthracis is the causative agent of anthrax disease, a serious disease that presents itself in the form of skin ulcers and systemic infections with a high mortality rate. This bacterium is dangerous to humans due to its virulence factors that help it infect a host organism. Much research in the field has focused on the importance of the plasmid-encoded anthrax toxins and capsule; both are critical for virulence. However, the goal of this project is to identify new chromosomal genes that also contribute to virulence in *B. anthracis*. Previous screening of a transposon library identified 11 *B. anthracis* mutants attenuated in infecting *Caenorhabditis elegans*, an invertebrate worm model. To further validate virulence phenotypes and prioritize transposon mutants for follow-up, a *Galleria mellonella* invertebrate infection model was used to assess survival of the 11 transposon mutants originally identified in *C. elegans*. One mutant, TN2, showed virulence attenuation in both models. TN2 has a disruption in a promoter region that we hypothesize controls two genes: a putative BNR repeat domain protein (2A) and a glycosyl-like 2 transferase family protein (2B). For my project, I attempted insertional mutagenesis to inactivate the 2A gene with the goal of confirming that this gene is linked to virulence, rather than unintended mutations elsewhere in the genome. Unfortunately, an insertional mutant disrupting 2A (Δ2A) was not able to be constructed. However, we still compared the ability of the wild-type and the original transposon mutant to survive exposure to hydrogen peroxide and determined that the transposon mutant is not susceptible to reactive oxygen species. This research identified a potential novel bacterial virulence factor and more information on its mechanism of action thus expanding our understanding of bacterial pathogenesis.

ACKNOWLEDGEMENTS

I would like to thank Dr. Shauna McGillivray, my research mentor for her support throughout my time at TCU and for sharing her passion for microbiology with me. I would also like to thank my committee members, Dr. Mikaela Stewart and Dr. Lisa Vanderlinden, for their feedback on my thesis and their support during my education at TCU. This project would not be possible without funding which was provided by the Science and Engineering Research Center. I would like to thank my lab mates have made the countless hours spent in the lab so enjoyable. Lastly, I would like to thank my family and friends for encouraging me and helping me to grow over the last four years.

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INTRODUCTION

Bacillus anthracis is the rod-shaped, gram-positive, spore forming bacteria responsible for causing anthrax, a serious infectious disease. Due to the relatively low incidence of anthrax and its ability to manifest in many ways, it is often difficult to initially diagnose. The routes of infection include cutaneous, gastrointestinal, and inhalation anthrax with cutaneous being the most common and inhalation being especially lethal.[1] When the skin is damaged, *B. anthracis* spores can enter human hosts and cause cutaneous infection. One of the common signs of a cutaneous anthrax infection is the presence of a black eschar. Patients with cutaneous anthrax have high rates of survivability due to the efficacy of treatment.[2] Inhalational anthrax occurs when *B. anthracis* spores are inhaled. These spores can evade post-phagocytic lysis in the macrophage and use the macrophage to travel through the lymphatic system, causing the infection to reach systemic levels rapidly. Inhalational anthrax infection is rare and typically presents with vague flu-like symptoms, making it initially difficult to diagnose. This allows the pathogen time to mount a system-wide infection, making inhalational anthrax especially deadly.[3]

B. anthracis is zoonotic, meaning it infects animals, which then can spread *B. anthracis* to humans and other animals they contact. However, anthrax is not spread through human-to-human contact. Most of the modern cases of anthrax involve recent exposure to animal products or infected livestock that have been exposed to this soil-dwelling bacterium.[2] When *B. anthracis* experiences harsh conditions or stress, it can form an endospore, which allows it to remain dormant for decades, becoming resistant to many harmful conditions and requiring few nutrients for survival. Due to the hardy nature of the endospores and the nonspecific symptoms, *B. anthracis* has been used as a bioweapon in the past and continues to be a threat. In 2001, in a case code-named "Amerithrax," anonymous letters laced with *B. anthracis* spores were sent to

individuals in the United States. Unfortunately, five Americans died due to exposure to the endospores and seventeen other individuals became infected.[4]

The genome of *B. anthracis* is composed of approximately 5,000 genes located on its circular chromosome and two plasmids: pXO1 and pXO2. Several major virulence factors associated with *B. anthracis* are located on its plasmids. The pXO1 plasmid encodes the lethal toxin and the edema toxin both of which interrupt immune signaling.[5] The pXO2 plasmid encodes a capsule. Due to the thick polysaccharide composition of the capsule, the host immune system has a difficult time recognizing the bacterium and thus is unable to effectively phagocytose it.[6] Therefore, the infection cannot be cleared efficiently. The strain of *B. anthracis* lacking the pXO2 plasmid is known as the Sterne strain. Without the pXO2 plasmid, the bacterium lacks much of its pathogenicity and is thus safe to study in a BSL-2 laboratory without the risks associated with studying the nonattenuated strain. Additionally, the Sterne strain has been used in vaccines.

There is a large body of evidence suggesting that both plasmids play an integral role in the virulence of *B. anthracis*. However, there has not been much investigation focused on the role of chromosomal genes in the virulence of *B. anthracis*.[7] To identify potential chromosomal genes involved in the pathogenesis of *B. anthracis*, Dr. Shauna McGillivray created a transposon library of *B. anthracis* Sterne mutants. Each of these mutants has a large section of DNA inserted randomly into the chromosomal genome. These mutants were initially screened using red blood cells to determine if any of the mutants had lost hemolytic activity. Bacteria lyse red blood cells as a method of sequestering iron as a nutrient, so a transposon insertion in a region needed to carry out this process would render this mechanism inactive. Through this screen, a *B. anthracis* mutant with a transposon interrupting the *clpX* gene was identified. Further investigation of this

gene confirmed that *clpX* plays a significant role in *B. anthracis* defense against the host immune system.[8]

This transposon library was next screened in a live animal model. Screening 5,000 mutants in a vertebrate model, such as mice, would be a logistical challenge and would lead to ethical concerns. Therefore, the invertebrate model organism *Caenorhabditis elegans* was selected. The innate immune system of *C. elegans* has many conserved defenses such as the production of reactive oxygen species and antimicrobial peptides. Additionally, *C. elegans* previously proved to serve as an effective host for *Pseudomonas aeruginosa* and *Staphylococcus aureus*.[9] Because of the small size of *C. elegans* and its ability to produce a large number of genetically identical offspring in a short amount of time, screening with *C. elegans* was a more efficient and ethical model. Of the 5,000 transposon mutants screened, 11 mutants showed attenuated lethality in *C. elegans*. One of these mutants, known as TN1, was determined to have an interruption in a tellurite-resistance gene, and it was further characterized and shown to have a role in virulence.[10] However, TN2-TN11 still remained uncharacterized.

This project aims to identify which of the remaining 10 transposon mutants, TN2-TN11, should be further investigated. To accomplish this, a screen with a second invertebrate model, *Galleria mellonella* was performed. Like *C. elegans, G. mellonella* has many conserved immune defenses such as the production of reactive oxygen species and antimicrobial peptides. However, the immune system of *G. mellonella* is more robust with phagocytic hemocytes present. Due to the small body size of *C. elegans,* the bacterial strains had to be ingested by the worms, making it difficult to ensure even pathogen exposure. *G. mellonella* has a larger body size and can be injected with specific amounts of the bacterial strains being investigated. Additionally, *G. mellonella* can be incubated at 37 ºC, which is the optimal temperature for bacterial growth.[11]

Mutants that are attenuated in both invertebrate infection models are more likely to harbor disruption in critical genes, and they will be prioritized for follow-up. This includes making independent mutations in the disrupted genes, confirmation of a virulence phenotype and investigation into possible mechanism of action.

METHODS

Bacterial strains and growing conditions

B. anthracis Sterne ($pXO1^+$, $pXO2^-$) served as the parental strain. *B. anthracis* Sterne transposon mutants (TN2-TN13) were taken from the McGillivray transposon library. *ΔclpX* was used as an attenuated strain.[8] Commercial MC1061 F^- *Escherichia coli* used in transformation were purchased from Lucigen. The methylation deficient *E. coli* strain used was GM2163. Unless otherwise noted, Brain Heart Infusion (BHI) media was used in all assays to grow the bacterial strains. All *B. anthracis* strains were grown at 37 ºC shaking, and all *E. coli* strains were grown at 30 ºC shaking.

Screening mutants in Galleria mellonella

The waxworms purchased from various online bait shops (rainbowmealworms.net and bestbait.com) were placed in the 4 ºC refrigerator in order to keep them dormant. The waxworms were then weighed, and those that were within the range of 180-230 mg were divided into groups of approximately 10. Overnight cultures of the transposon mutants, wild-type, and a known attenuated strain (Δ*clpX*) were grown using 1 colony in 3 mL of BHI. The following day, 100 µl of the overnight cultures were added to 3 mL of BHI to create day cultures, which were all grown to log phase as represented by an optical density of approximately 0.4. The bacterial cultures were then washed and resuspended in 3 mL of phosphate buffered saline (PBS). 500 µl of the washed culture was added to 500 µl of PBS to create a 1:2 dilution. The waxworms were then injected in the posterior cuticle with 10 µl of the diluted bacterial cultures with an automated pump and a 27-gauge needle. Once all worms were injected, they were placed in the 37 ºC incubator. The number of surviving waxworms were recorded after 24, 48, and 72 hours.

Starting inoculums were determined by making 10-fold serial dilutions of the 1:2 diluted log phase cultures. Colonies were counted and colony-forming units (CFU) were calculated approximately 16 hours after the plates were put in the incubator to ensure similar amounts of bacteria were injected into each waxworm.

Creating targeting plasmid

A region of homology to the gene of interest (2A) was cloned into the suicide plasmid (pHY304). To amplify a 350 base pair portion of the 2A gene, Polymerase Chain Reaction (PCR) was utilized. One colony of *B. anthracis* Sterne was added to 10 µl of sterile water and microwaved for 1 minute to free the DNA. Two 50 µl PCR reactions were set-up, each with 2.5 µl of the *B. anthracis* Sterne DNA, 2.5 µl of 10uM TN2/35G10 IM Fwd XhoI primer (Table 1), 2.5 µl of 10 µM TN2/35G10 IM Rev HINDIII primer (Table 1), 5 µl 10X PCR buffer, 5 µl 10x dNTPs, 2.5 µl of Taq polymerase, and 30 µl of sterile water. These mixtures were amplified via PCR. Gel electrophoresis was utilized using a 1% agarose gel, 1X TAE buffer, 15 µl of our PCR product with loading dye and gel green added, 10 µl of ladder A, and our amplified PCR product at 95 V for approximately 45 minutes to confirm that a portion of 2A was amplified. The remainder of the PCR product was stored at 4 ºC, and later purified using the Promega Wizard SV Gel and PCR Clean-Up System. The concentration of our purified DNA was determined to be 115 ng/µl using the nanodrop. To digest our PCR product, 40 µl of the PCR product was mixed with 1 µl of Xho I (restriction enzyme), 1 µl of HindIII (restriction enzyme), 5 µl of 10x 2.1 NEB buffer, 3 µl of sterile water. To digest our destination vector, 10 ug of 844 ng/µl pHY304 were added to 2 µl of Xho I, 2 µl of HindIII, and 5 µl of 10x 2.1 NEB buffer, and 28.2 µl of water. Both digest reactions were incubated at 37 ºC for 2-4 hours. To dephosphorylate the

ends of the destination vector, 1 µl of calf-intestinal phosphatase was added to the digested destination vector and incubated for an additional 30 minutes at 37 ºC. Using the Promega Wizard SV Gel and PCR Clean-Up System, the digested PCR product and the digested and dephosphorylated destination vector were purified. The nanodrop was then used to measure the concentration of both purified DNA products (PCR product = 30.3 ng/µl; plasmid = 16.6 ng/µl). To ligate the amplified section of the 2A gene to the digested pHY304 suicide plasmid, we added a 5:1 insert to plasmid molar ratio (aiming for approximately 100-200 total ng of DNA) to 2 µl of 10x ligation buffer, 1 µl of NEB DNA ligase, and 11.9 µl of sterile water to reach a 20 µl total reaction. This reaction was incubated overnight at room temperature to create the ligated plasmid before transformation into *E. coli.*

Transformation of E. coli

To transform *E. coli*, 50 µl of commercial electrocompetent Lucigen *E. coli* MC1061 was thawed in an ice bath. In a microcentrifuge tube, 1 µl of the ligation was added to 25 µl of the thawed MC1061 *E. coli*. In a second microcentrifuge tube, 2 µl of the ligation was added to 25 µl of thawed MC1061 *E. coli*. After sitting in the ice bath for 10 minutes, the mixtures were added to electroporation cuvettes and shocked at 1800 mV. All time constants were approximately between 4.0 and 5.0. Immediately following the shock, 950 µl of Lucigen recovery media was added to the electroporation cuvettes. The recovery media and bacteria mixtures were added to cell culture tubes and placed in the 30 ºC shaking incubator for 1 hour. Following incubation, the cultures were plated on Erm500 antibiotic plates and placed in the 30 ºC static incubator for approximately 2-3 days to monitor growth. The presence of the plasmid was determined using

colony PCR with pHY3065Fwd and PHY 3260 Rev primers (Table 1) followed by gel electrophoresis.

Overnight cultures were started using 1 colony of the transformed MC1061 *E. coli* in 3 mL of BHI with Erm500. The plasmid was purified using IBI Scientific High-Speed Plasmid Mini Kit**.** Electrocompetent transformation was repeated using GM2163 *E. coli* which are methylation deficient. SOC recovery media was used after shocking the cultures. The transformed bacteria was grown on Erm5 plates in the 30 ºC static incubator for 2-3 days. The presence of the plasmid was determined using colony PCR with pHY3065Fwd and PHY 3260 Rev primers (Table 1) followed by gel electrophoresis.

Electrocompetent transformation of B. anthracis Sterne

The parental Sterne strain was grown overnight in 3 mL BHI at 37 ºC shaking. In a 500 mL Erlenmeyer flask, 0.5 mL of the overnight culture was added to 50 ml 0.5% glycerol BHI and grown to and optical density of 0.6-0.8. The cells were then collected using a 500 mL sterile filter apparatus with at 0.2-0.5 um filter attached to a vacuum. The cells collected on top of the filter were washed by adding 25 mL cold electroporation buffer (1 mM HEPES 10% glycerol pH 7.0) on the cells and pipetting up and down. The cells were washed 3 times and then resuspended to 1/20th of the original volume. In two separate electroporation cuvettes 80 µl of the cell suspension and either 2.5 ul or 5.0 ul of the unmethylated plasmid at a concentration of 163.4 ng/ul (post-purification using IBI Scientific High-Speed Plasmid Mini Kit) were added together and kept on ice for 15 minutes. The bacteria were then shocked at 1800 mV and the time constants were recorded (between 4.0-5.0 ms). Immediately after shocking, 500 µl of BGGM (BHI containing 10% glycerol, 0.4% glucose, and 10 mM $MgCl_2$) recovery media was added to

the electroporation cuvette. The solution was then added to a sterile polypropylene tube and incubated at 30 ºC shaking for 1 hour. Following incubation, the cultures were plated on Erm5 plates and grown at 30 ºC for 2-3 days. The presence of the plasmid was confirmed by PCR using pHY3065Fwd and pHY3260 Rev primers (Table 1) followed by gel electrophoresis.

Insertional mutagenesis: selecting for integration

Overnight cultures of the transformed *B. anthracis* were grown at 30 ºC in Erm5 BHI to select for bacteria with the plasmid present. The next morning, 100 µl of overnight culture was added to 5mL Erm5 BHI and grown shaking at 37 ºC for 8 hours. The culture was then diluted 1:500; 1:1000, and 1:5000. 100 µl of each dilution was plated on Erm5 BHI plates and grown overnight at 37 ºC. pHY304 is unable to replicate at 37 ºC, so any bacterial growth is due to colonies with the plasmid integrated into the chromosome. Integration was confirmed by performing colony PCR with 2725 Fwd pHY and TN2A-IM-confirm-Rev-2 primers (Table 1) followed by gel electrophoresis.

Minimum inhibitory concentration (MIC) assay

To attempt to determine the function of the genes controlled by the promoter inactivated by the transposon in TN2, various MIC assays comparing TN2, *B. anthracis* Sterne, and known attenuated strains were performed. One colony of the bacterial cultures was grown overnight in 3 mL of antibiotic BHI in the shaking incubator at 37 ºC. The overnight cultures were diluted 1:20 and 100 µl of each were plated in a 96 well plate along with 100 µl of BHI with various concentrations of antimicrobial agents to achieve final concentrations of 200 µl in each well. The plates were incubated at 37 ºC for 16-20 hours and the optical densities were determined. Serial

dilutions of the 1:20 diluted log phase cultures were plated to determine the CFUs/ml of the starting inoculum.

Table 1: List of primers used in PCR amplification.

Primer Names	Primer Sequences
TN2/35G10 IM Fwd XhoI	ACA GTC TCG AGA CGC TCG AAG CTC TTG GG
TN2/35G10 IM Rev HINDIII	GAC TAA GCT TAC TCG TTT ATA AAT GTT ATC CCT GAG ATA CTA
TN2/35G10 IM confirm Rev	CTT CAA GAC TTG GAA TAT CTT TTA TAG GCT
TN2B IM Fwd (XhoI)	ACA GTC TCG AGA TTG TTA CAA ATC AGG CCG ATG
TN2B IM Rev (HindIII)	GAC TAA GCT TCA GTA AAC CCT ACT CTT CTT CCT TT
TN2 IM confirm Rev	GTT GCT TCC CGT CAT AAA TTG A
TN2A-EV-Fwd (SalI):	AGC TGT CGA CTG TGT AAA GAT AGT AGA TAA AAC GAG TGG TA
TN2A-EV-Rev (SalI):	TCG TGT CGA CCG TTG TGT GTT ACA ACT CTC ACC
TN2A Fwd	GAA CTG ATA GAT TTG GTT GGG CTG
TN2A-IM-confirm-Rev-2	CAA GCC CAA CCT TTA CGC CG
2725 Fwd pHY	GGT ATA CTA CTG ACA GCT TCC AAG GAG
pHY3065Fwd	ACG ACT CAC TAT AGG GCG AAT TGG
pHY 3260 Rev	GCG GAT AAC AAT TTC ACA CAG G

RESULTS

Transposon mutant screen in *Galleria mellonella* determines a mutant of interest

One of the primary goals of this research was to determine if the *B. anthracis* transposon mutants which showed lethality attenuation in *C. elegans*,[10] repeated the attenuated phenotype in *G. mellonella*. To investigate this, Bella Kouretas, another undergraduate researcher, and I performed a series of *G. mellonella* survival assays. The strains tested were all grown to log phase as measured by an optical density of approximately 0.4, washed in PBS, and diluted 1:2 with PBS. Groups of approximately 10 waxworms weighing between 180-230 mg were injected with 10 µl PBS or the various prepared 1:2 diluted bacterial strains including *B. anthracis* Sterne, a known attenuated strain (Δ*clpX*), and the transposon mutants TN2-TN13. To ensure similar amounts of bacteria were injected, the CFUs/ml were calculated for each of the 1:2 dilutions of the strains tested and approximately equal concentrations of bacteria were present. The worms were incubated at 37 ºC, and after 24, 48, and 72 hours, the number of surviving worms were recorded. Living waxworms move and are a creamy yellow in color. As the waxworms begin to die, black spots appear, and the waxworms turn completely black when they die (Figure 1).

Figure 1: ***G. mellonella* larvae infected with *B. anthracis* Sterne and PBS.** Dead waxworms after injection with wildtype bacteria (top petri dish) and living waxworms injected with PBS (bottom petri dish).

The survival assay was carried out using wild-type Sterne *B. anthracis*, phosphate buffered saline (PBS), a previously attenuated strain (Δ*clpX*), and the various transposon mutants. The percent of worms which survived following injection can be seen in be seen in Table 2.

Table 2: Results of *G. mellonella* survival assays

Strain (# worms)	Percent Survival at 24 hours	Percent Survival at 48 hours	Percent Survival at 72 hours
WT (76)	23.7	17.1	14.5
PBS (74)	78.4	75.7	71.6
Δ*clpX* (66)	62.1	54.5	51.5
TN2 (28)	60.7	53.6	53.6
TN3 (28)	39.3	39.3	35.7
TN4 (28)	35.7	25.0	25.0
TN5 (22)	18.2	9.1	9.1
TN6 (22)	9.1	4.5	4.5
TN7 (22)	9.1	0.0	0.0
TN8 (34)	50.0	32.4	26.5
TN9 (12)	50.0	33.3	25.0
TN10 (34)	38.2	32.4	29.4
TN11 (12)	0.0	0.0	0.0
TN12 (12)	50.0	0.0	0.0
TN13 (12)	0.0	0.0	0.0

After 72 hours, only 14.5% of the worms injected with wild-type Sterne *B. anthracis* survived; whereas 71.6% of the worms injected with PBS (the negative control) survived. Some death in the waxworms injected with PBS is to be expected due to the trauma associated with injection. Waxworms injected with a previously identified attenuated strain, Δ*clpX*, had a 51.5% survival rate. Of note, 53.6% of *G. mellonella* injected with TN2 survived after 72 hours, which is comparable to the attenuation seen in the Δ*clpX* strain. Additionally, when survival curves between WT and TN2 were compared, the survival rate with TN2 was statistically different than the survival rate with WT (figure 2).

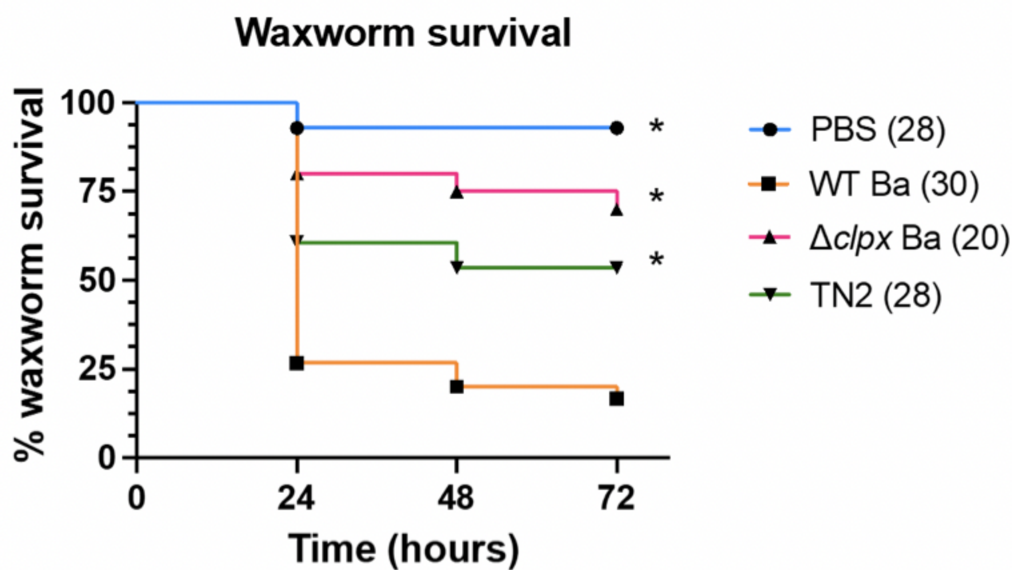


Figure 2: TN2 is attenuated in *G. mellonella* survival assay. The number of surviving worms was recorded after 24, 48, and 72 hours. The total number of waxworms injected can be seen in parentheses. *Indicates statistically significant differences ($p < 0.01$) in survival from WT using the log-rank test.

Interrupting 2A via insertional mutagenesis

In Franks et al, the genomes of transposon mutants TN1-TN11 were sequenced to determine the site of transposon insertion.[10] In TN2, the transposon inserted into a promoter, which we hypothesized controlled the expression of an operon containing two genes: putative BNR repeat domain protein (2A) and glycosyl-like 2 transferase family protein (2B) (Figure 3).

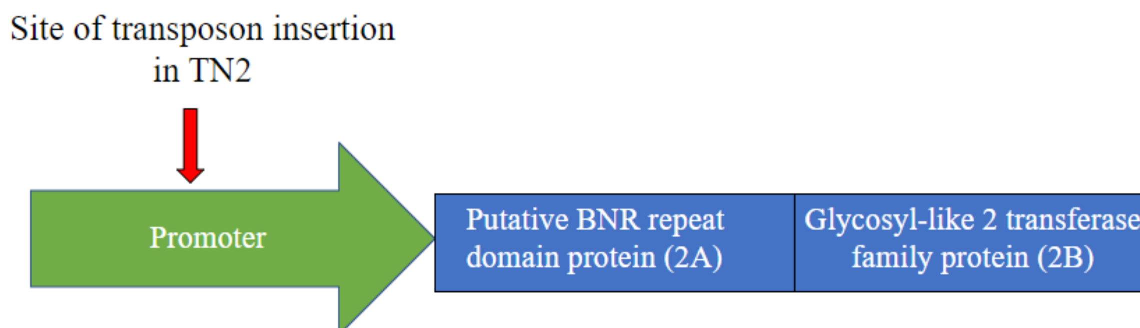


Figure 3: **Genome structure of affected genes of mutant TN2**. This schematic is a representation of the approximate location of the transposon insertion in the TN2 mutant.

To determine whether the decreased virulence seen in the transposon mutant, TN2, was due to the inactivation of gene 2A or gene 2B, we attempted to create insertional mutants, interrupting each of these genes individually. The creation of these insertional mutants would also confirm that the attenuation seen in TN2 is due to the transposon insertion and not a random mutation elsewhere in the genome. I focused on constructing the insertional mutant Δ2A, which would render inactive the gene coding for the putative BNR repeat domain protein. The previously mentioned undergraduate student, Bella Kouretas, focused on creating Δ2B. To construct the insertional mutant, a mutagenic plasmid was created by ligating a 350 base pair sequence of the 2A gene into a temperature sensitive plasmid with an Erm resistance gene, known as pHY304. When this plasmid is transformed into *B. anthracis,* the region of 2A present in this plasmid has homology with the 2A gene present in the chromosome of *B. anthracis* Sterne. This allows for homologous recombination to take place, where the plasmid inserts itself into the host chromosome, rendering the 2A gene inactive (Figure 4).

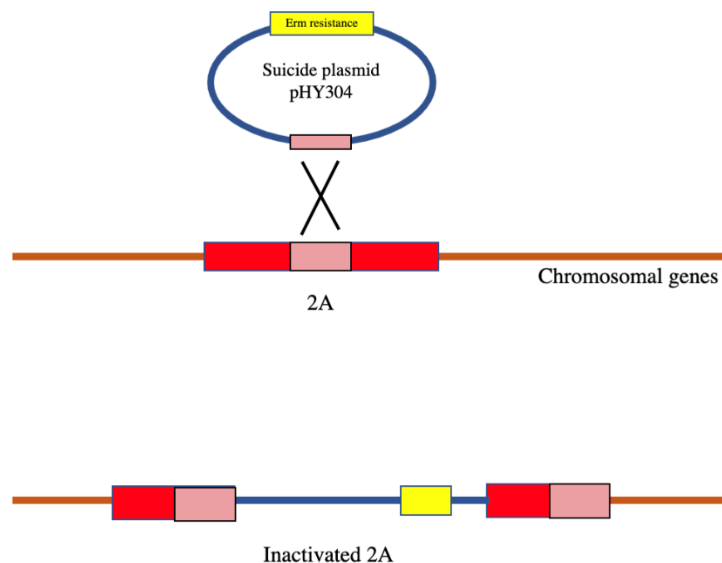


Figure 4: Schematic of creating the Δ2A insertional mutant in *B. anthracis* Sterne. The suicide plasmid pHY304 (blue circle) with a region of homology (pink) to the 2A gene (red and pink) in *B. anthracis* was used to attempt to interrupt the 2A gene in the host chromosome (orange) via homologous recombination.

MC1061 F- *E. coli* and GM2163 methylation-deficient *E. coli* were consecutively transformed with the targeting plasmid to have a sufficient amount of unmethylated plasmid to later transform *B. anthracis* Sterne. Following each of these transformations, *E. coli* colonies grew on Erm500 plates, suggesting that the bacteria had taken up the plasmid. The presence of the plasmid was also confirmed after each of these transformations via colony PCR using pHY304 Fwd and Rev primers (Table 1, Figure 5). This amplified a portion of the pHY304 plasmid (blue line) encompassing the multiple cloning site where the 2A insert is located. If the region of homology inserted into the plasmid, approximately 550 base pairs should be amplified. If it failed to insert, approximately 200 base pairs should be amplified. The colonies used in Lanes 2 and 4 had successful ligation of the region of homology to the suicide plasmid (Figure 5). Their bands show that a larger region of DNA was amplified compared to the empty plasmid seen in Lane 5. The absence of a band in Lane 3 suggests that this particular colony was not successfully transformed therefore lacking the plasmid.

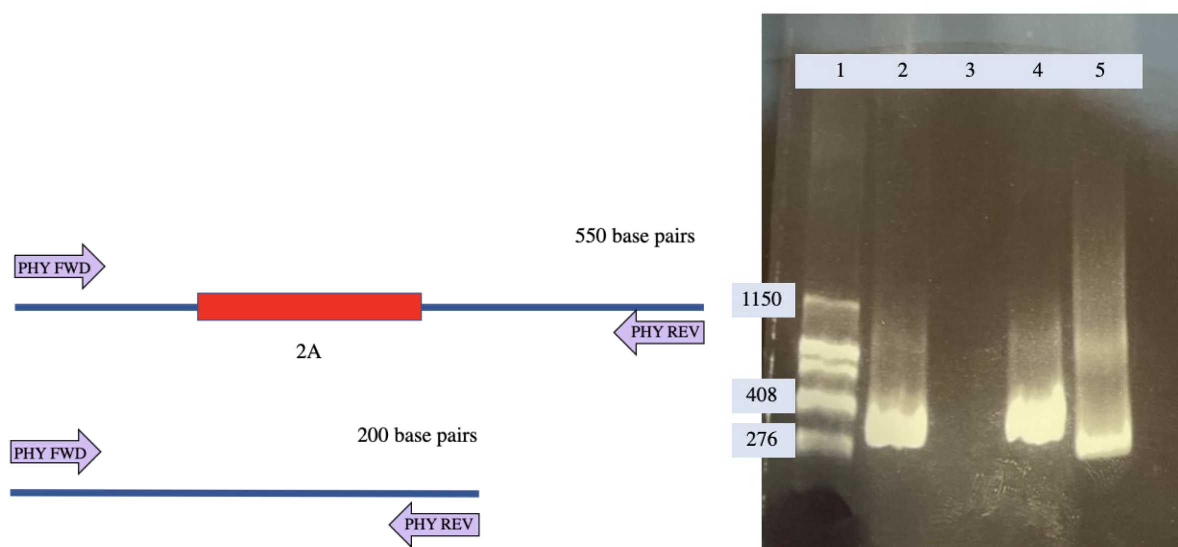


Figure 5. Construction of targeting plasmid. (A) Schematic of PCR amplification strategy confirming targeting plasmid formation. Primers pHY304 Fwd (PHY FWD) and pHY304 Rev (PHY REV) were used to amplify a portion of the pHY304 plasmid (blue line) encompassing the multiple cloning site where the 2A insert is located. (B) PCR of *E. coli* colonies following transformation. The gels used in our lab consistently run fast compared to the ladder. Lane 1 contains Ladder A, Lanes 2 and 4 contain DNA from successfully transformed *E. coli* colonies, Lane 3 contains DNA from an untransformed *E. coli* colony, and Lane 5 contains DNA from the empty pHY304 plasmid.

After confirming successful transformation, we purified the plasmid and transformed *B. anthracis* Sterne. Following *B. anthracis* Sterne transformation, colonies grew on Erm5 BHI plates, suggesting the presence of the Erm resistance gene originally from the plasmid. Because pHY304 has a temperature sensitive origin of replication, it will only replicate at 30 ºC. To select for bacteria with the plasmid integrated into the middle of the 2A gene, the bacteria were grown at 37 ºC. Following the temperature shift, colonies grew on the Erm5 BHI plates at 37 ºC, suggesting that the plasmid had integrated into the chromosome in these colonies. If this occurred, it would have placed the 5,000 base pair plasmid in the middle of the 2A gene (see schematic in figure 4). In this case, PCR using the pHY304 2725 Fwd and TN2A confirm Rev primers were used in an attempt to amplify a region of DNA including a portion of the integrated plasmid (blue) and a portion of the host genome (orange). This should have yielded a band of approximately 600 base pairs (Figure 6). However, colony PCR resulted in a band sizes of less than 200 bp, most likely representing primer dimers (Figure 6). Due to the lack of a positive control in this experiment, these results suggest that either the plasmid failed to integrate into the host chromosome or that the PCR did not work properly.

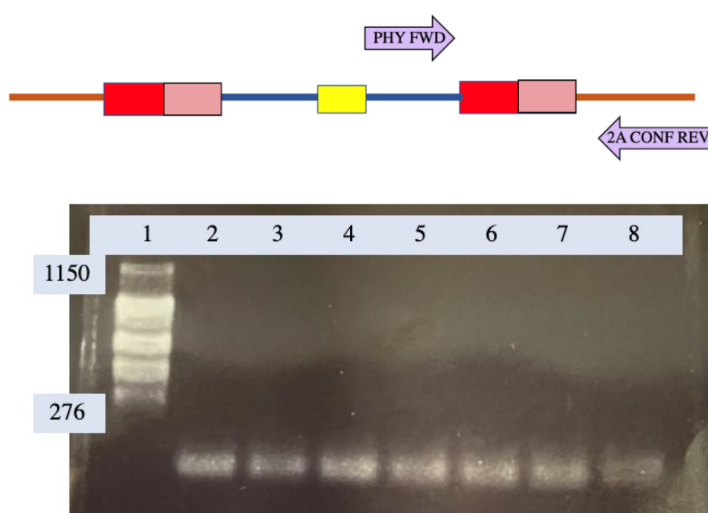
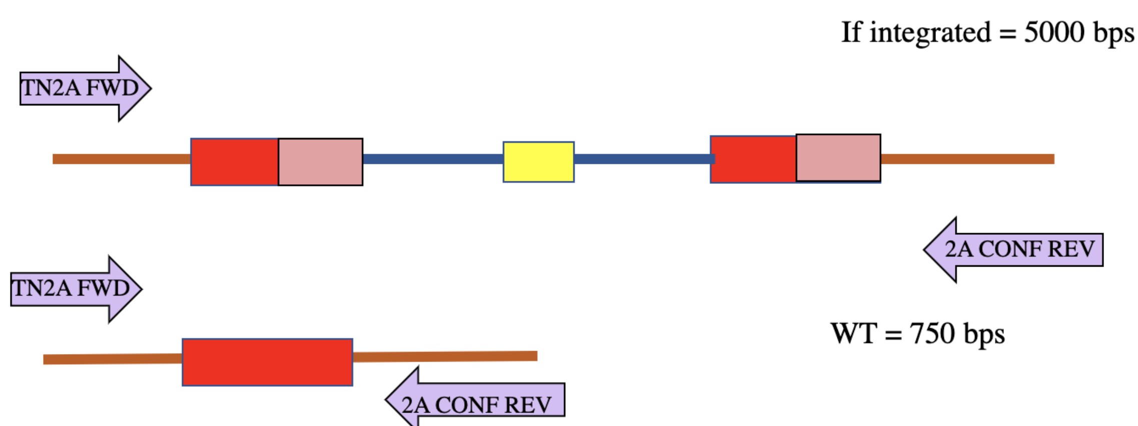


Figure 6: Lack of plasmid integration into host chromosome. (A) First schematic of PCR amplification designed to confirm plasmid integration into *B. anthracis* Sterne**.** Primers used include pHY304 2725 Fwd (PHY FWD) and TN2A confirm reverse primers (2A CONF REV). (B) PCR of colonies post-temperature shift. Lane 1 contains Ladder A. Lanes 2-8 contain DNA from various *B. anthracis* colonies.

Therefore, we repeated the temperature shift a second time with two passes through fresh antibiotic culture. Again, colonies grew at 37 ºC on Erm5 BHI plates, suggesting that the plasmid had integrated into the chromosome in these colonies. However, the colony PCR using pHY304 2725 Fwd and TN2A confirm Rev primers still showed that the plasmid failed to integrate into the host chromosome. Using a different approach to colony PCR, TN2A Fwd primer (binds upstream of the potential insertion) and TN2A IM confirm reverse primer (binds downstream of the potential insertion) were used in an attempt to amplify a section of DNA over 5,000 base pairs in length which included a portion of the host chromosome (orange) and the entire plasmid (blue). If the plasmid truly integrated into the host chromosome, the resulting sequence should be over 5,000 base pairs. This is too large to be amplified; therefore, a lack of a band would confirm integration. However, there are bands present in lanes 2-7 (the band in lane 2 is present but faint) which contain amplification from colony DNA. These bands match the length of the band in lane 8, which contains amplification from WT DNA, suggesting that the plasmid once again did not integrate into the host chromosome (Figure 7).



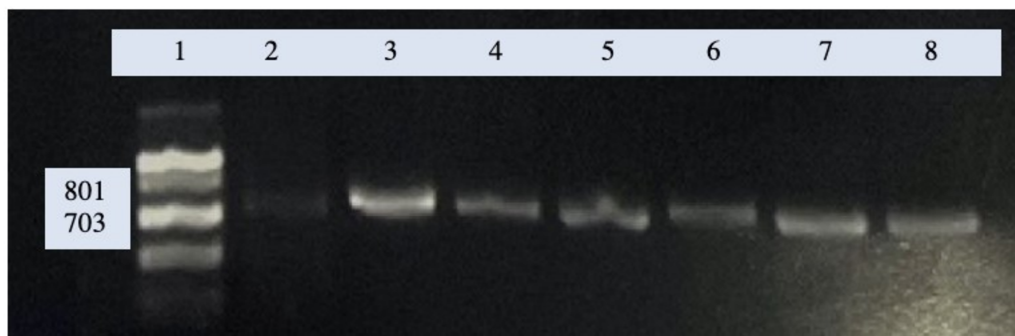


Figure 7: Lack of integration into host chromosome. (A) Second schematic of PCR amplification to confirm plasmid integration using primers TN2A FWD and 2A CONF REV. **(**B) PCR using TN2A Fwd and TN2A IM confirm reverse primers. Lane 1 contains Ladder A. Lanes 2-7 contain colony DNA from various *B. anthracis* colonies grown on Erm5 after the temperature shift. Lane 8 contains untransformed WT *B. anthracis Sterne* colony DNA.

Characterizing the function of TN2 via MIC assays

Although an insertional mutant disrupting the 2A gene was unable to be constructed, further investigation was carried out to characterize the phenotype of TN2. Because reactive oxygen species are utilized to prevent infection by both *G. mellonella* and mammalian hosts, we decided to perform a hydrogen peroxide minimum inhibitory concentration (MIC) assay. If TN2 showed decreased growth compared to WT when exposed to hydrogen peroxide (H_2O_2), this would suggest that the gene interrupted by the transposon in TN2 may be involved in the neutralization of reactive oxygen species. In this assay, levels of bacterial growth were measured in various concentrations of H_2O_2. In 0% H_2O_2, there is no difference of growth between WT, 11F11 (a *B. anthracis* strain known to be susceptible to H_2O_2), and TN2, confirming that all three strains have similar amounts of growth in BHI alone (Figure 8). When exposed to 0.00875% H_2O_2, 11F11 shows a decrease in growth that is statistically significant. However, TN2 shows no difference in growth compared to the WT, suggesting that the genes interrupted in TN2 are not involved in the neutralization of reactive oxygen species.

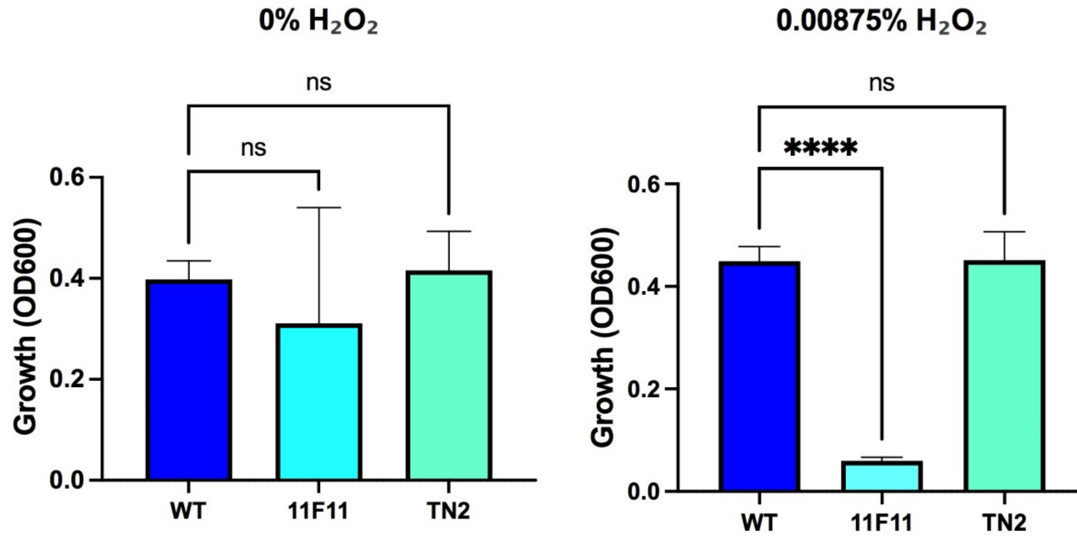


Figure 8: TN2 is not susceptible to hydrogen peroxide. WT, 11F11, and TN2 were grown in 0% H_2O_2 and 0.00875% H_2O_2. Data is presented as mean +/- one standard deviation from combined results of three assays. ****Indicates statistical significance by one-way ANOVA with the Dunnett's post-hoc test, $p<0.005$

DISCUSSION

Our goal was to use a *G. mellonella* survival assay to determine which of the remaining transposon mutants (TN2-TN11) showed attenuation in two independent invertebrate infection models. This lends additional support for the likelihood that the genes interrupted by TN2 may be important in virulence. Throughout this project, we had some challenges with the *G. mellonella* infection model. The data from approximately four of our trials was unable to be used because our controls did not yield the expected results. Many of the worms injected with PBS died. To ensure we had healthy worms, we modified the protocol. Before carrying out the waxworm survival assay, test injections were performed. Larvae were either injected with 10 µl of PBS or poked with a 27-gauge needle, neither of which should not kill healthy worms. After 24 hours, the number of surviving worms was counted. If a significant percentage of worms died, we did not proceed with the assay, as this was an indicator that the batch of worms was not healthy enough to survive the trauma associated with injection. Additionally, we hypothesized that the variety of temperatures that the *G. mellonella* larvae experience may have led to the excess death seen in these trials. Therefore, we eliminated their refrigeration at 4 °C. Leaving the worms at room temperature prior to injection, showed results closer to what we expected in our controls. Although, this made the worms more difficult to inject, since they were not dormant, having accurate controls outweighed this added challenge. Despite these challenges, we found that the TN2 mutant was consistently attenuated in its ability to infect the waxworms relative to the WT strain.

The site of insertion of TN2 is known to be in a promoter region [10] which we hypothesize controls two genes: BNR repeat domain protein (2A) and glycosyl-like 2 transferase family protein (2B). From there, insertional mutants were constructed to inactivate these two genes

individually. Although Δ2A was unable to be constructed, my fellow undergraduate student investigator, Bella Kouretas, was successfully able to construct Δ2B. Further research by future students working in this lab should be carried out to investigate how these genes may be involved in virulence in *B. anthracis*.

Due to the proximity of 2A and 2B to each other and their location on the same coding strand, we assumed that they were both part of an operon controlled by the promoter interrupted by the transposon in TN2. In the future, this hypothesized structure would be important to confirm through an RNA expression assay. In this assay, the RNA levels of both 2A and 2B would be measured in both wild-type and TN2. If the promoter controlled both genes, there would be decreased expression of 2A and 2B in TN2 compared to wild-type *B. anthracis* Sterne. However, if only 2A showed decreased expression in TN2, this would indicate that these genes do not actually operate in an operon and that the promoter only controls the expression of the BNR repeat domain protein.

My project primarily focused on gene 2A, which is a bacterial neuraminidase repeat (BNR) domain protein. These proteins contain short, repeated sequences of amino acids.[12] After searching the literature, there is not much known about the function of BNR repeat domains, as they are found in many non-homologous proteins.[13] The repeats have been found in glycosyl hydrolases,[13] which is relevant as 2A is located next to a glycosyl-like-2 transferase family protein. Creating insertional mutants is an important step in the process of characterizing the function of these genes. Unfortunately, an insertional mutant was unable to be constructed for Δ2A. However, this should be attempted again in the future. The 2A region of homology that was inserted into the suicide plasmid was only approximately 350 base pairs. Another undergraduate student was able to create an insertional mutant interrupting 2B using a region of

homology of approximately 450 base pairs. When re-attempting to create Δ2A, using a larger region of homology may increase the likelihood for insertion.

After creating Δ2A, it is critical to repeat the waxworm survival assay to compare survival of *G. mellonella* after being injected with WT *B. anthracis*, Δ2A, and Δ2B. If either of the insertional mutations show attenuation similar to that seen in TN2, this would suggest that the gene interrupted in the insertional mutant could be a virulence factor. If neither of the insertional mutants show increased survival compared to the wild-type, this would indicate that the gene interrupted in that insertional mutant is not important in virulence and perhaps the other gene is the virulence factor. It is also a possibility that both genes need to be inactivated for the phenotype to be present. Therefore, a double insertional mutant could be constructed in which both 2A and 2B are inactivated. To further confirm whether 2A or 2B is important in virulence, complemented strains TN2+2A, TN2+2B, and TN2+2A+2B could be constructed. The 2A gene alone, the 2B gene alone, or both 2A and 2B together would be inserted into a plasmid. Eventually, the TN2 strain of *B. anthracis* could be transformed with each of these plasmids and then reinjected into the waxworms in search of restoration of the wild-type phenotype. This would help to isolate whether 2A, 2B, or the combination of the two together is important in virulence.

After further confirming the attenuation of TN2 and isolating which specific gene is important to virulence, the next step would be to characterize how the hypothetical operon containing 2A and 2B creates the attenuated phenotype. *G. mellonella* has many similar host immune defenses to humans such as the production of reactive oxygen species and antimicrobial peptides, and the presence of both opsonizing proteins that recognize conserved microbial patterns and phagocytic hemocytes.[14] Since TN2 showed attenuation in *G. mellonella*, one of the

genes in the inactivated operon may target one of those host immune defenses. To test this, minimum inhibitory concentration (MIC) assays using H_2O_2 were performed. There was no significant difference in growth between WT and TN2, suggesting that the genes interrupted in TN2 are not important in the neutralization of reactive oxygen species. Additional MIC assays could be conducted comparing the growth of *B. anthracis* Sterne, TN2, Δ2A, Δ2B, and known attenuated strains when exposed to other conserved host defenses such as antimicrobial peptides. This would assist in characterizing the role that the promoter and genes 2A and 2B play in virulence. If *B. anthracis* Sterne has a higher MIC for a particular antimicrobial substance than TN2 or the insertional mutants, this would suggest that a higher concentration of the antimicrobial would be required to inhibit the growth of *B. anthracis* Sterne. Thus, the gene rendered inactive in these strains may be important in protecting the bacteria from that particular antimicrobial produced by the host.

In conclusion, this research has successfully identified a potential novel chromosomal virulence gene in *B. anthracis.* Given the rise in antibiotic resistance amongst bacterial pathogens, this research is important because, after further characterization, BNR repeat domain protein or glycosyl-like 2 transferase family protein could serve as new antibiotic targets directed at treating anthrax. As various bacterial species tend to have regions of homology, these antibiotics could be used to target a wide variety of bacterial infections beyond anthrax, helping to combat the rise in antibiotic resistance.

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