INVESTIGATION OF THE GLYCOSYL-LIKE 2 TRANSFERASE FAMILY PROTEIN AND ITS ROLE IN *BACILLUS ANTHRACIS* VIRULENCE

by

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ABSTRACT

The gram-positive bacterium, *Bacillus anthracis,* is responsible for the deadly disease Anthrax. *B. anthracis* is dangerous due to virulence factors, or defenses the bacteria uses to infect a host. We hope to better understand how this bacterium interacts with its hosts by studying the genes necessary for virulence. Bacterial mutants, which have a change in their genetic sequence, sometimes show reduced ability to cause disease in a host. Studying these mutants helps us understand the bacteria's infection method. Previously, our lab created a library of mutants using a technique called transposon mutagenesis and then screened these transposon mutants for phenotypes linked to decreased virulence. This resulted in the identification of 11 transposon mutants that were less effective at causing disease in the nematode *Caenorhabditis elegans*[1]. To prioritize these mutants, we tested them using a second infection model, the caterpillar *Galleria mellonella*. *G. mellonella* is an ideal model due to its optimal size for injection, conserved innate immune defenses, and previous success as an infection model for *B. anthracis*[2]. We found that only one of these 11 mutants, TN2, had reduced virulence in both *C. elegans* and *G. mellonella*. Future research will focus on confirming the genetic change in this mutant and determining the mechanism by which it contributes to infection. This could lead to new antibiotic targets in the future.

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INTRODUCTION

Bacillus anthracis is a spore-forming, gram-positive bacterium, and the causative agent of anthrax disease. *B. anthracis* is usually found in the soil as a stable and dormant spore.[3] The tough outercoat of the bacterial spore allows it to persist in the environment for long amounts of time resisting high heat, UV light, gamma rays, and some antimicrobials. Because of this characteristic, anthrax is transmitted zoonotically when grazing herbivores contact the robust spores in the ground. The disease is not passed from human to human but can jump from animals to humans. Animal to human transmission occurs when a person is working with infected livestock or around contaminated animal products[4]. There are three routes in which anthrax can infect a host: cutaneous, inhalational, and gastrointestinal. Cutaneous anthrax is the most common form, making up over 90% of all human cases. The bacterium enters through a lesion on the skin and will form a small pimple, which over time turns into a black eschar. In contrast, inhalational anthrax is not as common but is highly lethal without treatment. The disease first presents itself with mild flu-like symptoms but in the second stage of the disease, septic shock and coma may occur which in most cases lead to death. Inhalational anthrax is not caught as early on due to the more moderate symptoms in the beginning, so it is often too late for a person to be treated in the later disease stages[5]. Fortunately, if caught early enough, the use of appropriate antibiotics makes it so that the mortality rate for anthrax is less than 1%[6]. In 2001, Anthrax and its causative agent *B. anthracis* became an increasingly important area of study due to its use as a biological warfare agent. Letters were sent out to various public figures in the United States with the intention of them contracting anthrax by inhaling the *B. anthracis* endospores found in the envelope. A total of 22 people contracted anthrax and five died[7]. Since this bacterium had been used as a weapon, researchers attention shifted to better understanding

B. anthracis' method of infection so we could quickly and efficiently treat infected patients in the future.

Pathogens infect and colonize hosts using characteristics we refer to as virulence factors. These virulence factors differ between bacteria and understanding their mechanisms can bring us insight on how to stop or slow certain bacterial infection. The most well understood *B. anthracis* virulence factors are found on two plasmids: pXO1 which codes for toxins, and pXO2 which codes for a bacterial capsule. Without one of these plasmids, the bacterium has trouble evading the host immune system and considered to be less virulent. The *B. anthracis* Ames strain is what we consider to be fully virulent, this means the bacteria possesses all its defenses against host immune systems including both plasmids listed above[8]. In our lab, we use a less virulent or attenuated strain which cannot cause disease in a human host. This is referred to as *B. anthracis* Sterne strain. This strain is attenuated in a host organism, meaning it is less able to fight off immune defenses due to the fact it is missing the pXO2 plasmid and cannot express a capsule. This capsule makes it harder for the host immune system to recognize the bacteria and clear the bacteria from the body. Although we understand the role of the plasmid genes that *B. anthracis* uses to evade host immunity, it is important to analyze the whole genome of the bacterium in addition to the plasmids. There are over 5,000 chromosomal genes in the *B. anthracis* chromosomal genome and over 600 genes of unknown function, some of which may contribute to the bacterium's virulence[6].

In order to better understand the role of the chromosomal genes in *B. anthracis*, our lab constructed a mutant library using transposon mutagenesis[9]. A transposon library consists of thousands of bacterial mutants, each mutant with an interruption in a different part of the genome. Changes in phenotype resulting from these genetic disruptions can then be screened.

Our lab has conducted several screens for virulence-associated phenotypes. The *clpX* gene was identified through screening the transposon mutants for a loss of hemolytic and proteolytic phenotypes. ClpX is critical for virulence because when mice were infected with a *B. anthracis* strain containing a disrupted clpX gene (Δ*clpx*), all of them survived while every mouse infected with a WT *B. anthracis* Sterne strain died after five days[9]. Following the characterization of clpX, our lab performed a separate screen on the transposon mutant library. This screen utilized *Caenorhabditis elegans* as an infection model in the above assay, a worm model known to have a highly conserved innate immune system with various host defenses[10]. This identified the *yceG* and *yceH* genes (also known as TN1) as important in bacterial defense against tellurite and against reactive oxygen species (ROS) such as hydrogen peroxide. In addition to TN1, 10 other transposon mutants were attenuated in the *C. elegans* model which are referred to as TN2 through TN11. Although TN1 was the most attenuated transposon mutant in this *C. elegans* screen with a mortality rate of less than 20%, TN2-11 are also attenuated in the host and are promising targets of characterization in our research[1].

To better understand the rest of the TN mutants, we want to confirm their attenuation in an additional animal model, specifically the wax worm model *Galleria mellonella.* Although both *C. elegans* and *G. mellonella* have a conserved innate immune system and are inexpensive, *G. mellonella* is a more optimal invertebrate model for screening transposon mutants. *G. mellonella* is slightly larger in size making it easier to inject with bacterial cultures. It is also easier to maintain because it can be incubated at 37°C which is the optimal temperature for *B. anthracis* growth. Lastly, this wax worm model contains innate defenses such as production of ROS and phagocytic hemocytes with even more homology to mammals than *C. elegans* has[11]. If these mutants show a phenotype of lower mortality rate in the *G. mellonella* invertebrate model

in addition to the *C. elegans* model, there is a greater chance that these genes will be truly important for *B. anthracis* virulence. Once we have identified the transposon mutants which are the most attenuated in *G. mellonella*, we will construct an insertional mutant of *B. anthracis* where we will disrupt the same gene that is being interrupted by the transposon insertion. This will allow us to confirm that the observed phenotype in the model organism is due to the specific transposon insertion and not another miscellaneous mutation in the bacterial genome. Next, we will perform various in vitro screens using other host defenses, beginning with hydrogen peroxide, which is a ROS used by mammalian hosts to defend against pathogen infection. Our goal is to identify novel genes that are important in *B. anthracis* virulence.

EXPERIMENTAL PROCEDURES

Bacterial strains and growing conditions

In this project, the *Bacillus anthracis* Sterne strain was used. For the entirety of this research unless otherwise noted, *B. anthracis* Sterne strain and each transposon mutant were grown in brain heart infusion (BHI) media at 37°C shaking. Two different *E.* coli strains were used in this research for transformation. Commercial MC1061 *E. coli* was purchased from Lucigen and GM2163 methylation deficient *E. coli* was also used. *E. coli* strains were grown at 30°C shaking in liquid BHI media.

Survival assay in wax worm model Galleria mellonella

Eleven transposon mutants identified in prior research[1] were tested for attenuated virulence in the *G. mellonella* model. *G. mellonella* worms purchased from Rainbow Mealworms were stored at 4°C to slow down their movement for more optimal injection conditions. *G. mellonella* were weighed and separated into a group weighing 190-220 mg, worms outside of this range were not used in the assay. Between 10-12 worms were used for each condition. In each trial, 3-4 transposon mutants were tested. Each trial consisted of a wild-type *B. anthracis* control, a 1x Phosphate Buffer Saline (PBS) control and a 4D5 mutant control which is a known previously attenuated strain. Each different strain was streaked out on a BHI plate two days before an assay and grown overnight at 37°C under static conditions. Overnight cultures were made from the streak plates by placing a single colony in 3 mL of BHI and grown overnight under 37°C shaking conditions. The next morning, 100 µL of the overnight culture was transferred into 3 mL of BHI and grown to log phase (optical density 400) at 37°C shaking conditions. This took around 2-3 hours. 1000 µL of bacteria was then transferred into a 1.5 mL microcentrifuge tube and centrifuged for 5 minutes at full speed. Supernatant was removed and

bacteria were washed and then resuspended in 1000 µL of 1x PBS. The bacteria were diluted 1:2 with 1x PBS before being injected into the worms. Using an automated pump and a 27-gauge needle, 10 µL of the above bacterial dilution was injected into each worm at the posterior end of the body. Worms were incubated at 37°C for a total of 72 hours and the total number of worms which survived were recorded at 24 hours, 48 hours, and 72 hours. After injection of worms, the 1:2 bacterial dilution was plated using 10-fold serially dilution to determine the starting cfu/mL.

Construction of pHY304 plasmid with TN2B sequence

Before amplification of glycosylase like 2 family protein in *B. anthracis* via PCR (polymerase chain reaction), streak plates were made on BHI agar and grown at 37°C under static conditions to obtain genomic (colony) DNA. The next day, about half a colony from each control and experimental strain was put in 10 µL of water in a PCR tube and microwaved for one minute. This served as our template DNA for PCR. Two separate 50 µL PCR reactions were run using the forward primer pHY3065 Fwd and reverse primer pHY3175 Rev (table 1). Each reaction consisted of 5 µL of 10x dNTPs, 2.5 µL of 10 µM forward primer and 2.5 µL of 10 µM reverse primer, 2.5 µL colony DNA, 2.5 µL Taq polymerase, 5 µL 10x PCR buffer. After completion of PCR, 15 µL of loading dye was added to the 50 µL of PCR product and 15 µL of PCR product was run through an agarose gel using gel electrophoresis. The band was compared to standard ladder measurements to confirm that the TN2B section of the genome was amplified. The remaining PCR product was stored at 4°C before being purified using Promega gel and PCR cleanup (Wizard SV Gel and PCR Clean-Up System). The concentration of the DNA product was checked to ensure the DNA sequence was concentrated enough.

The PCR product and destination plasmid pHY304 were then digested separately. When digesting the PCR product, 40 µL of PCR product, 1 µL of XhoI restriction enzyme, 1 µL of

HINDIII restriction enzyme, 5 µL of 2.1 10x NEB buffer, and 3 µL of water were added to a 1.5 mL microcentrifuge tube for a total volume of 50 µL. When digesting the destination vector pHY304, 10 µL of 844 ng/µL pHY304 pure plasmid, 1 µL of XhoI restriction enzyme, 1 µL of HINDIII, 5 µL of 2.1 10x NEB buffer, and 37.1 µL of water was added to reach a total volume of 50 µL. Both microcentrifuge tubes left to incubate at 37°C for 2-4 hours. Then, the ends of pHY304 were dephosphorylated by the addition of 1 µL of CIP to the digest tube with pHY304, the digest with pHY304 was incubated for 30 minutes at 37°C. We then purified both digests using the Promega purification kit and determined the concentration of the pure, digested, DNA. Next, we ligated our TN2B sequence into pHY304 by adding 4 µL of our pHY304 vector at a concentration of 16.6 ng/µL, 1.1 µL of TN2B insert at a concentration of 30.3 ng/µL, 2 µL of 10x ligation buffer, 1 µL of NEB DNA ligase, and 11.9 µL of water to get a total volume of 20 µL in a 1.5 mL microcentrifuge tube. The ligation mixture was left to incubate at room temperature for 15 minutes.

Transformation of pHY304 + TN2B insert into electrocompetent Escherichia coli

We next transformed the plasmid and the insert into the MC1061 F- *E. coli* (Lucigen). We thawed the electrocompetent *E. coli* on ice. Once thawed, we added 2 µL of the ligation from the previous step and 50 µL of the *E. coli* into a 1.5 mL microcentrifuge tube and let it set for an additional 10 minutes in the ice bath. All 52 µL of the *E. coli* and the ligation mixture were transferred to an electroporation cuvette and shocked at 1800 mV in the electroporator (Eppendorf). 950 µL of recovery media was added directly to the electroporation cuvette. Bacteria, ligation, and SOC recovery media mixture was transferred to a 5 mL culture tube and incubated for one hour shaking at 30°C. Three separate dilutions were measured and plated all on erm500 BHI plates. The first plate had 100 µL of the bacteria mixture in the culture tube only,

the second plate had 100 μL of a 1:2 dilution of the bacteria and BHI, and the third plate had 100 μL of a 1:4 dilution of the bacteria and BHI. The plates were incubated at 30°C overnight. The following day PCR was performed to confirm the pHY304 plasmid contained the TN2B insert. Primers used were pHY3065 FWD and pHY3175 REV as a control on the empty pHY304 plasmid and TN2B forward and TN2B reverse primer on 3 separate colonies of *E. coli* containing the plasmid and insert. After confirmation of plasmid with insert using PCR, *E. coli* cultures were grown in erm 500 BHI media. 4 mL each in 16 mL culture tubes were grown overnight at 30°C. A glycerol stock of the *E. coli* with plasmid was frozen down for later use, 250 μL of 80% glycerol was added to 750 μL of the remaining overnight culture. We then isolated the plasmid from the remainder of the culture using a commercial miniprep kit (IBI).

Following transformation of pHY304 and TN2B insert into MC1061 *E. coli*, we then transformed the plasmid and insert into a second electrocompetent *E. coli* strain GM2163. We began by using the frozen *E. coli* stock with glycerol of 100 μL to grow an overnight culture in 3 mL erm500 BHI at 30°C shaking. The plasmid was purified, then this overnight culture was miniprepped the next morning with the same IBI kit as above. We repeated the above steps, beginning with thawing electrocompetent GM2163 on ice, mixing it with the miniprepped *E. coli* above, shocking it, and plating it in the same dilutions above. The electrocompetent GM2163 were plated on erm500 plates and left to grow at 30°C. Colony PCR was performed again with the same primers as above to confirm the pHY304 plasmid with the TN2B insert had successfully been transformed into the GM2163 electrocompetent *E. coli*. Plasmid was then purified from GM2163 as described above.

Transformation of pHY304 plasmid with TN2B insert into electrocompetent Bacillus anthracis

Next, we transformed our plasmid and insert into *B anthracis*. First wild type *B. anthracis* was streaked out on a BHI agar plate and left to incubate at 37°C overnight. The next night, one colony of *B. anthracis* was put into 3 mL of BHI with 0.05% glycerol and incubated at 37°C shaking conditions overnight. The following day, 0.5 mL of the overnight culture was transferred to a 500 mL Erlenmeyer flask with 50 mL BHI and 0.05% glycerol. The mixture was incubated in shaking conditions of 200 rpm at 37°C for about 3-4 hours until it reached an optical density of 0.6 – 0.8. Cells were then collected using a 500 mL sterile filter (Cell Treat Products) by applying vacuum. Once cells had been separated from media, the cells on the filter were washed with 25 mL of ice-cold electroporation buffer. Cells were washed with buffer a total of 3 times and then resuspended in 2.45 mL of electroporation buffer and then placed on ice in a culture tube. 5 µL of miniprepped clean DNA from the GM2163 *E. coli* transformation and 80 µL of *B. anthracis* washed cell suspension was added to a 0.1 cm gap electroporation cuvette and left on ice for an additional 15 minutes. The electroporation cuvette was put in the electroporator and shocked at 1.8 – 2 kV and 500 µL of BGGM recovery media was added to the cuvette immediately after. The BGGM recovery media contains 1.25 mL 80% glycerol, 200 µL 20% glucose, 100 µL 1 M MgCl2 and 8.45 mL of BHI. The contents of the electroporation tube were transferred to a culture tube and incubated at 30°C shaking conditions for an hour. 100 µL of the culture was plated two times on erm5 and 150 µL of the culture was plated two times on erm5 for a total of four plates. The plates were incubated at 30°C static conditions. Colony PCR was performed to confirm the pHY304 plasmid with the TN2B insert had successfully been transformed into the electrocompetent *B. anthracis*. PHY3065 forward and pHY3260 reverse primers were used on an empty pHY304 plasmid as a control and TN2B forward and reverse

primers were used on colony DNA to confirm presence of pHY304 plasmid with insert in *B. anthracis*.

Construction of insertional mutant in Bacillus anthracis

Once PCR confirmed the presence of pHY304 inside competent *B. anthracis*, overnight cultures of the electrocompetent *B. anthracis* were grown with 3 mL of erm5 BHI and a single colony. Growing conditions were 30°C shaking. The next morning, 100 µL of the overnight culture of competent *B. anthracis* is transferred into 3 mL of erm5 BHI and grown at 37°C shaking conditions for eight hours. The culture is diluted in erm5 BHI in 1:500, 1:1000, and 1:5000 and then plated on three different erm5 BHI agar plates to incubate overnight at 37°C. Once colonies appeared, PCR was used to confirm that the pHY304 plasmid with the TN2B insert has integrated into the *B. anthracis* host genome. Colony PCR is done using pHY3065 forward primer and IM confirm reverse TN2B primer on the colony DNA and pHY3065 forward and pHY3260 reverse on empty pHY304 plasmid as a control.

Minimum inhibitory concentration of hydrogen peroxide assay

We first wanted to test the insertional *B. anthracis* mutant with disruption of TN2B for attenuated bacterial defenses against H_2O_2 hydrogen peroxide. Streak plates were made of WT *B. anthracis* on BHI agar, 11F11 mutant on kan50 BHI agar, our original TN2 transposon mutant on BHI agar, and our newly constructed Δ2B insertional mutant on erm5 BHI agar. All plates were grown at 37°C static conditions overnight. Overnight cultures were made from each plate with each different bacteria in appropriate antibiotic media at 37°C shaking conditions. The next day 100 µL of each of the overnight cultures were added to 3 mL of BHI and grown at 37°C shaking until log phase (OD 0.4) was reached for about 3 hours. Then the log phase culture of each different strain was diluted 1:10 in BHI liquid media. 100 µL of this 1:10 dilution was

added to each appropriate row in a 96-well plate. 100 μL of varying concentrations of H_2O_2 consisting of 0%, 0.00218%, 0.004375%, 0.00875%, and 0.0175% were added to each well with each different strain. Colony counts were performed from the day cultures to make sure there was similar amounts of CFU in each different strain day culture. Bacterial growth against the H_2O_2 was quantified by looking at optical density at a wavelength of 600 nm. In two of the three assays, O/N cultures of the different strains were diluted 1:20 in BHI and used in the assay in place of the 1:10 dilution of day cultures.

Table 1: Primers Used in PCR Confirmation and Construction of Insertional Mutant Δ2B

Primer name	Primer sequence
pHY3065 Fwd	5'– ACG ACT CAC TAT AGG GCG AAT TGG – 3'
pHY3175 Rev	5' – CCG CTC TAG AAC TAG ATC CCC – 3'
TN2/35G10 IM Fwd XhoI	5' – ACA GTC TCG AGA CGC TCG AAG CTC TTG GG – 3'
TN2/35G10 IM Rev HINDIII	5' – GAC TAA GCT TAC TCG TTT ATA AAT GTT ATC CCT GAG ATA CTA – 3'
TN2/35G10 IM confirm Rev	5' – CTT CAA GAC TTG GAA TAT CTT TTA TAG GCT – 3'
TN2B IM Fwd (XhoI)	5' – ACA GTC TCG AGA TTG TTA CAA ATC AGG CCG ATG – 3'
TN2B IM Rev (HindIII)	5' – GAC TAA GCT TCA GTA AAC CCT ACT CTT CTT CCT TT – 3'
TN2 IM confirm Rev	5' – GTT GCT TCC CGT CAT AAA TTG A – 3'

RESULTS

Screening of transposon mutants in Galleria mellonella infection model

Transposon mutants obtained from research in McGillivray et. al (2014) were tested in groups of 3-4 mutants. Each group contained three controls: (1) a phosphate buffer saline (PBS) control which was used to make sure the act of injecting the worms was not contributing to worm death, (2) a wild-type (WT) positive control in which we injected the wax worms with *B. anthracis* Sterne strain and should lead to a low survival rate, and (3) a Δ*clpX* strain of *B. anthracis,* in which the *clpX* gene known to contribute to virulence is disrupted, and should yield a higher survival rate in the wax worms. Groups of *G. mellonella* were all similar weights to confirm that the worm survival rate is due to the mutant and not because of differences in bacterial injection to worm weight ratio. Each test group consisted of between 10-12 total wax worms. Bacterial strains were diluted and injected into the wax worms. The initial bacterial concentration up until the injection of the worms was similar in each test group. After injection, the worms were incubated at 37°C for three days. The worm survival percentage was documented after 24 hours, 48 hours, and then 72 hours.

Table 2: Survival of *G. mellonella* After Injection with Transposon Mutants TN2-TN13

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
Δclpx (66)	62.1	54.5	51.5
4D5 (10)	10.0	10.0	10.0
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

Table two shows the combined percent survival of each mutant and of the controls. The PBS worms were included in the assay to confirm that the act of injecting the worm was not lethal, so this group showed a higher survival percentage. All the following survival rates were recorded after a total of 72 hours. The WT control showed a very low overall survival rate of 14.5%. In comparison, the Δ*clpx* mutant showed much higher survival of 51.5% than the WT injection group due to its attenuated virulence. The wax worms injected with the TN2 mutant had the highest survival percentage of 53.6% amongst all the other mutants. TN3 worms had a 35.7% survival percentage and TN4 worms had a 25% survival. TN5, TN6, and TN7 had much lower survival percentages of 9.1%, 4.5%, 0%, respectively. Finally, mutants TN8 through TN13 had survival percentages as follows: 26.5%, 25%, 29.4%, 0%, 0%, and 0%. Of all mutants, TN2 showed the most promise with the highest combined rate of survival, which was statistically different from the survival of worms injected with WT (figure 1). Therefore, we chose this mutant to further investigate and characterize.

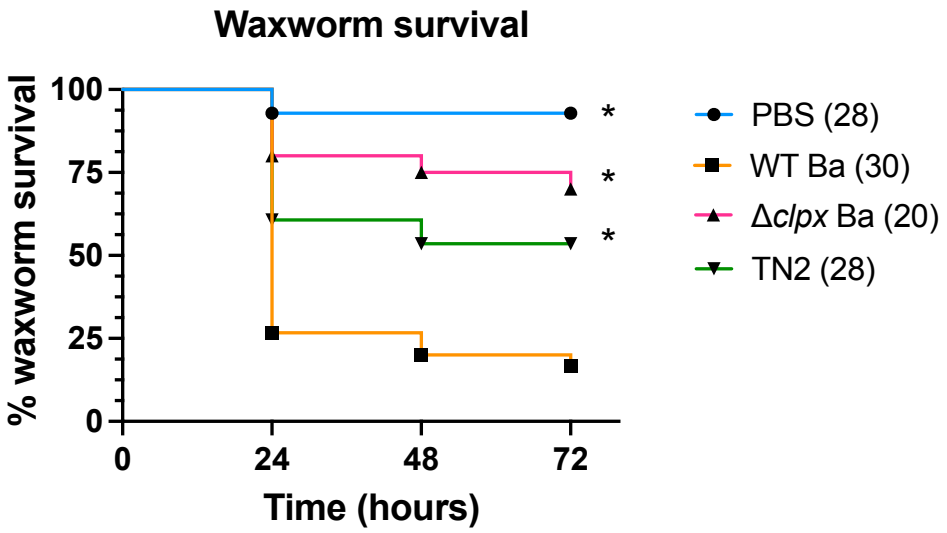


Figure 1: TN2 Transposon Mutant Shows Attenuation in *G. mellonella.* The graph shows the combined results of two independent tests. Total number of worms in each group are indicated in parentheses. *Indicates p<0.01 from survival with WT *B. anthracis* using the log rank test.

Construction of insertional mutant Δ2B in Bacillus anthracis

After analysis of the location of the transposon mutant TN2 in the *B. anthracis* genome, we hypothesized that the insertion in TN2 mutant is in a promoter region which potentially controls an operon comprising two different genes (Fig. 2). The genome annotation shows that the first gene in the putative operon encodes for the BNR repeat family protein. The second gene in the operon codes for a putative glycosyl-like 2 family protein. To determine whether either of these genes contribute to virulence, we decided to construct two different insertional mutants, the first one where the BNR repeat family protein is interrupted in *B. anthracis*. This is the first protein coding gene in the operon, so we refer to this mutant as Δ2A and my peer Lauren Klingemann investigated this portion of the genome and attempted to construct this mutant. The second insertional mutant contains an interruption in the glycosyl-like 2 family protein in the *B. anthracis* genome. We refer to this mutant as Δ2B and this will be the focus of my investigation.

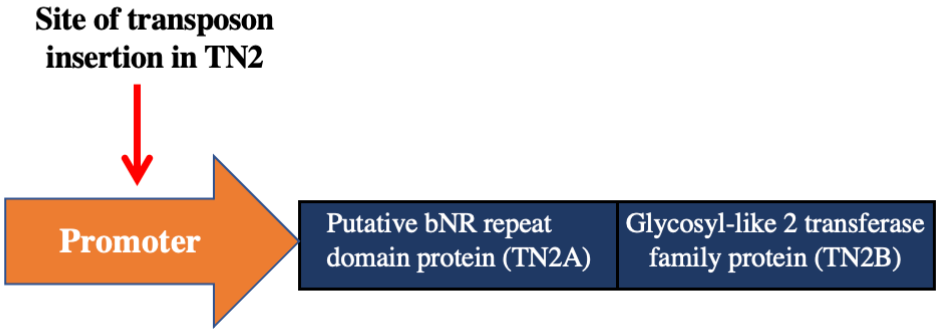
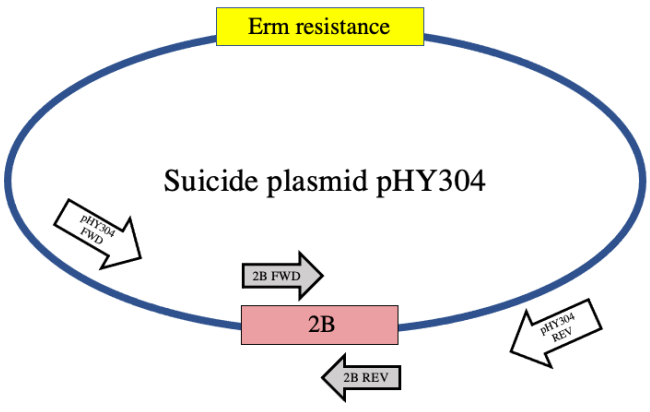
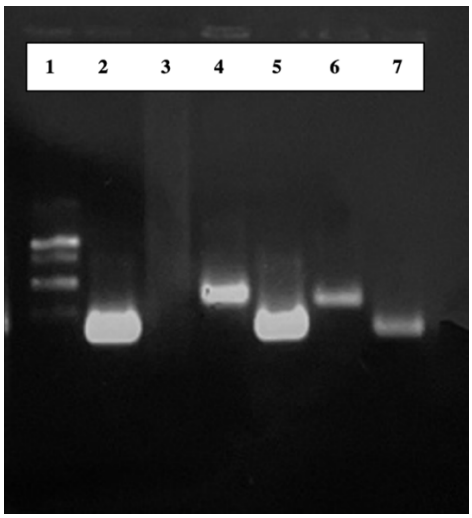


Figure 2: TN2 Insertion is in a Promoter Region. Sequencing of TN2 genome shows that the transposon insertion is in a promoter region which controls two protein coding genes, a putative BNR repeat domain protein (TN2A), and a glycosyl-like 2 transferase family protein (TN2B).

To construct this mutant, we used homologous recombination to generate a single recombination event in the middle of the putative glycosyl-like 2 family protein. We began by subcloning the DNA. We placed a 400-bp piece of homologous sequence from the middle of the glycosyl-like 2 transferase gene on a plasmid vector, pHY304 (Fig 3A). Then we transformed two different electrocompetent *E. coli* strains with the plasmid containing the TN2B sequence. Two different *E. coli* strains were used in transformation, the first strain MC1061 *E. coli* has high transformation efficiency and is ideal for cloning and the second strain, GM2163 *E. coli,* does not methylate the plasmid DNA, which prevents degradation in *B. anthracis* in later steps.



3A)



3B)

Figure 3: Construction of Targeting Plasmid. A) A pHY304 plasmid vector contains a temperature sensitive origin of replication, an antibiotic resistance gene against erythromycin represented in yellow, and a region of homology to a portion of the glycosyl-like 2 family protein in the *B. anthracis* genome. **B)** Lane 1 is ladder, lane 2 shows WT *B. anthracis* colony with TN2B forward and reverse primers. Lane 3 contained the pHY304 empty plasmid vector with pHY3065 Fwd and pHY3175 Rev primers but there was no amplification. Lanes 4 and 6 show a GM2163 colony using pHY3065 Fwd and pHY3175 Rev. Lanes 5 and 7 show a GM2163 colony with TN2B Fwd and Rev primers. All primer sequences are listed in table 1.

Figure 3 shows the results of PCR confirming that the electrocompetent GM2163 *E. coli* had taken up the pHY304 plasmid containing the TN2B sequence. Lanes 4 and 6 confirm that the *E. coli* had taken up the pHY304 plasmid. We know this because we used pHY3065 Fwd and pHY3175 Rev primers which amplify the plasmid and got a band of the expected size. This band did not run as far down on the gel as the band in lanes 5 and 7 because the primers in these lanes amplify only the inserted sequence and do not contain any plasmid DNA (see schematic in 3A) and are therefore shorter and will run faster. This amplification shows that the *E. coli* has taken up the plasmid containing the TN2B sequence.

After this, we created electrocompetent *B. anthracis* which would take up our plasmid containing a sequence homologous to 2B and insert in the chromosome in the middle of the 2B gene thus disrupting it. The bacteria were made competent by washing with an electroporation buffer, mixing them with purified pHY304 plasmid containing 2B, shocking them, and then growing up the new electrocompetent *B. anthracis* colonies. After confirming that the plasmid had been taken up by *B. anthracis* the next step was to select for colonies that had plasmid integration through a temperature shift screen. By first growing the potential *B. anthracis* insertional mutants in 30°C and then transferring them to 37°C, we were left with colonies that took up and successfully integrated the plasmid into their chromosomal genome. Additionally, because the plasmid contains an antibiotic resistance gene to erythromycin, bacteria without the plasmid will also not grow on antibiotic media. The pHY304 plasmid also contains a temperature

sensitive origin of replication which means that at 37°C and on an erm5 plates, *B. anthracis* colonies without plasmid integration will not grow because these plasmids will not be able to replicate at high temperatures (Fig. 4).

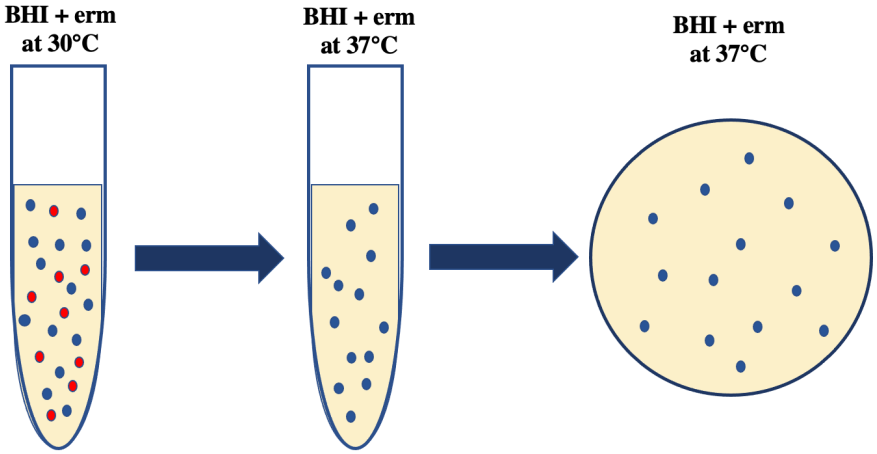
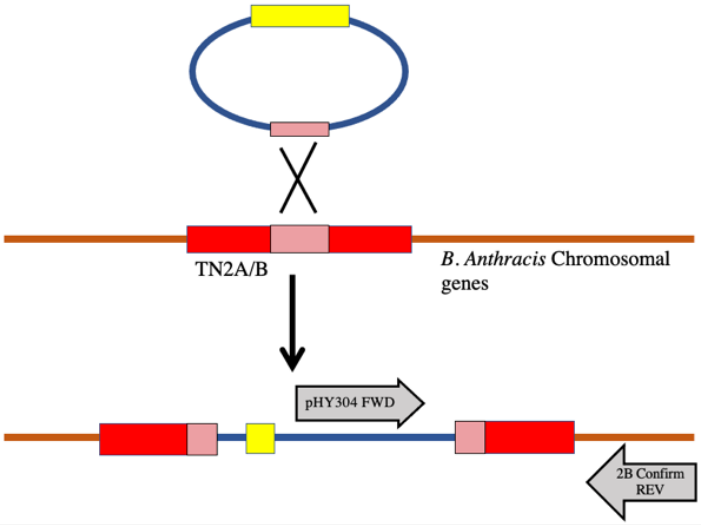
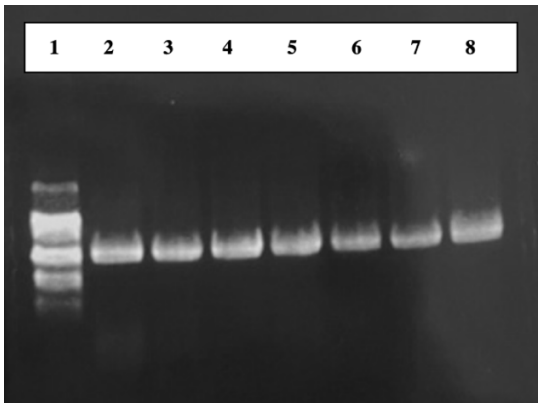


Figure 4: Selection of Insertional Mutants using Antibiotic Media and Temperature Sensitivity. A day culture was first grown at 30°C, red circles represent bacteria without plasmid integration, blue colonies represent *B. anthracis* insertional mutants. In tube 2, part of the culture in tube 1 was transferred to fresh BHI with erythromycin (erm5) and a new tube and grown at 37°C. The culture was then plated on an erm5 BHI plate and incubated at 37°C.

Colonies that grow after the screen contain a disruption in the glycosyl-like 2 family protein because the pHY304 plasmid inserted in the middle of the glycosyl-like 2 family protein gene through homologous recombination, hypothetically rendering this gene non-functional (Fig. 5A). Figure 5B shows PCR confirmation of the *B. anthracis* colonies with a disruption in 2B gene. The bands should be around 500 base pairs because the primers are amplifying from the beginning of the plasmid to the end of the glycosyl like 2 family protein sequence.



5A)



5B)

Figure 5: Construction of Insertional Mutant Δ2B. A) Target vector pHY304 inserted into the 2B gene in *B. anthracis* through homologous recombination. **B)** This PCR shows that the pHY304 plasmid with TN2B sequence has integrated into the *B. anthracis* genome, interrupting the TN2B sequence, we refer to this strain as Δ2B. pHY30427 forward primer and TN2B confirm reverse primer were used for each lane.

After successful construction of an insertional mutant Δ2B, we wanted to test this strain against various host defenses to better understand the role of glycosyl-like 2 transferase family protein and its mechanism of virulence. We began with a minimum inhibitory concentration assay using hydrogen peroxide, which is a reactive oxygen species produced by the mammalian innate immune systems. 22

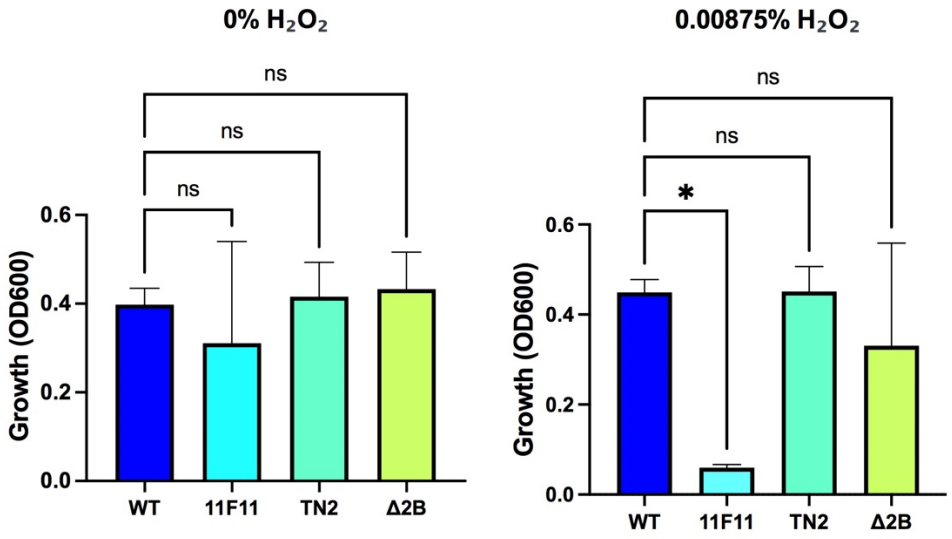


Figure 6: Growth of Transposon and Insertional Mutants in H_2O_2. Wild-type *B. anthracis*, 11F11, our transposon mutant TN2, and our insertional mutant Δ2B are grown in 0% and 0.00875% H_2O_2 and BHI solution. Data is presented as mean +/- SD from combined results of three assays. *Indicates statistical significance by one-way ANOVA with the Dunnett's post-hoc test, $p<0.05$.

The minimum inhibitory concentration assay is used to assess the lowest concentration needed to inhibit growth in different strains. The concentration used which inhibited growth of the known susceptible strain 11F11 but did not inhibit growth of WT was 0.00875% H_2O_2. 11F11 is used as a control because in previous lab findings, 11F11 has a mutation making it more susceptible to H_2O_2. Based on a total of three assays, there was no statistical significance in susceptibility between Δ2B and WT *B. anthracis* and there was also no similarity between 11F11 and Δ2B (Fig. 6). This tells us that the 2B gene is not important in *B. anthracis* defense against H_2O_2 because loss of the gene did not create a difference in phenotype in comparison to WT.

Table 3: Survival Assay Using Insertional Mutant Δ2B

Strain (# worms)	Percent survival at 24 hours	Percent survival at 48 hours	Percent survival at 72 hours
PBS (20)	80.0	75.0	75.0
WT (20)	50.0	30.0	25.0
Δ2B (20)	15.0	15.0	15.0
TN2 (20)	45.0	15.0	15.0
ΔclpX (20)	85.0	75.0	70.0

Finally, we wanted to use our insertional mutant Δ2B in a waxworm survival assay to confirm if the 2B gene is contributing to *B. anthracis* virulence as we had hypothesized. This assay was conducted with the same procedure as the previous assays in figure 1. Figure 7 shows a survival assay with PBS control, WT *B. anthracis*, Δ*clpx,* our original attenuated transposon mutant TN2, and Δ2B our constructed IM mutant. After a total of 72 hours, worms injected with PBS had a survival rate of 75%, worms injected with WT had a 25% survival rate, 15% survival rate for Δ2B, 15% for TN2, and 70% for Δ*clpx* (table 3). Based on our results, neither TN2 nor Δ2B, our experimental strains, show attenuation in a live host. Both strains show a lower survival rate in comparison to WT and no significant difference in survival in comparison to WT. These results are not consistent with our previous findings in figure 1 nor are they consistent with our hypothesis that 2B or glycosyl-like 2 family transferase protein is contributing to virulence in *B. anthracis*.

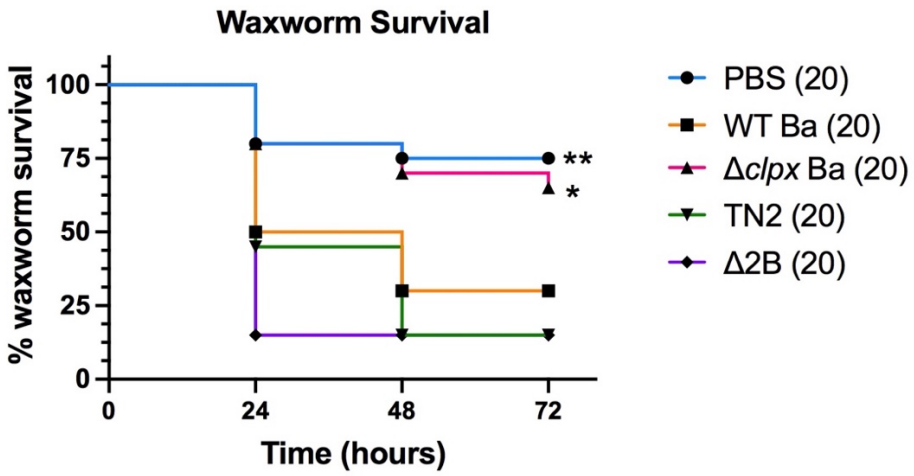


Figure 7: Δ2B Survival Assay in *Galleria Mellonella*. The graph shows the combined results of two independent tests. Total number of worms in each group are indicated in parentheses. *Indicates $p < 0.01$ from survival with WT *B. anthracis* using the log rank test.

DISCUSSION

We know that antibiotic resistance in bacteria is on the rise, and as of 2014 around 700,000 people globally die from antimicrobial resistance. This number is predicted to rise to 10 million deaths per year by 2050 which emphasizes the importance of investigation into therapeutic targets[12]. In this project, we are focusing on further identifying and characterizing chromosomal genes revealed by transposon mutants attenuated in *G. mellonella*. If these genes are homologous in other bacterial species, this research could provide widespread insight into protection against a variety of bacterial infections and allow us to keep up with the rise in antibiotic resistant bacteria. In addition to antibiotic targets, investigation into virulence factors can also give us new vaccine targets as well. Currently, a vaccine for anthrax exists but it is not widespread because supplies are limited in the United States and the efficacy of the vaccine is still being analyzed. The vaccine is given in six doses and uses the attenuated Sterne strain in many cases[13]. Depending on the type of anthrax; inhalational or cutaneous, various antibiotics such as ciprofloxacin, doxycycline, ampicillin, penicillin, vancomycin, and others can be given and are beneficial to one's health outcomes[6]. Fortunately, anthrax has effective therapeutic mechanisms, but this is not the case for all bacterial infections which makes bacterial virulence a very important region of study.

Previously in our lab, 5,000 transposon mutants were screened in *C. elegans* and 11 mutants were found to be attenuated in this model[6]. Because other researchers had already characterized TN1 and the importance of the interrupted gene, tellurite resistance proteins, for *B. anthracis* virulence, our project focused on narrowing down mutants TN2-TN11 to find the interrupted genes that contribute the greatest to virulence. In comparison to the other transposon mutants, TN2 was the only mutant attenuated in *G. mellonella* as well as *C. elegans* (Fig. 1). We

originally expected to see multiple transposon mutants show attenuation in the in-vitro screen with *G. mellonella* due to their previous attenuation in *C. elegans*, but this was not the case. Regardless, attenuation in both invertebrate models made it more likely that TN2 harbored a mutation in a gene relevant to virulence.

Our mutant of interest, TN2, is interrupted in a promoter region which we hypothesize controls a gene coding for putative BNR repeat domain protein and a gene coding for the glycosyl-like 2 transferase family protein. Due to their proximity, we hypothesize that these two genes are a part of a bacterial operon so we are interested in seeing how either gene or both genes together may contribute to *B. anthracis* virulence. Because TN2 transposon is inserted in a promoter region, it is important to investigate which gene controlled by the promoter is contributing to the attenuated phenotype during in vivo screens. This means it is not only important to characterize 2B in our research but 2A as well. Because the genes likely function on their own, it is important to investigate the loss of which gene results in the phenotype of lower worm death. For this reason, my research partner and I each chose one of the two genes, 2A and 2B to focus on. Unfortunately, we were only successful in creating the 2B mutation and future research will prioritize the construction of a Δ2A mutant by other means.

Although we do not have extensive information on the action of the glycosyl transferase encoded in 2A, we do know that glycosyltransferases may be important in some pathogenic bacteria because they help to create glycoproteins which help the bacteria to adhere to cell surfaces, form biofilms, and generally evade the host immune system[14]. It is possible that this glycosyl-like protein contributes to *B. anthracis* virulence in this way or via other mechanisms so

our next goal after prioritization is to characterize this gene and its function specifically in this bacterium.

To do this we wanted to test the TN2 mutant and our constructed insertional mutant Δ2B against various host defenses such as antimicrobial peptides and reactive oxygen species. We were able to test these strains against H_2O_2 through the completion of a minimum inhibitory concentration (MIC) assay. This assay revealed that the glycosyl-like 2 transferase gene was not involved in countering the immune system's release of reactive oxygen species like H_2O_2 so we can eliminate this as the potential mechanism for virulence in 2B. In the future, we hope to test antimicrobial peptides in an MIC assay to better understand the mechanism of the 2B gene.

Surprisingly, when we repeated our survival assay in *G. mellonella* using our constructed Δ2B mutant and TN2 original mutant, our results did not support our original findings. In our original assays (Fig. 1) we saw that TN2 was attenuated in a host, but in our later assays (Fig. 7) TN2 did not seem to be attenuated in our host organism and did not have a significant difference in survival rate in comparison to WT. Additionally, our Δ2B mutant was also not attenuated in the waxworm host, we can conclude that 2B is likely not a gene contributing to *B. anthracis* virulence. There are several reasons why we hypothesize that our results in figure 7 are not consistent with figure 1. The first is that throughout this research different worm companies have been used because in certain assays control groups were not working properly and we saw a very high percentage of worm deaths in all groups. Using a different brand of wax worm could be a contributor to this. Additionally, in our first several assays, the waxworms were refrigerated upon arrival to our lab and only taken out 15 minutes prior to injection. This was done to slow the worm movement and allow for a more optimal injection condition. After many assays, we

began to see a very low percent survival so we stopped refrigerating the worms and would leave them at room temperature prior to injection. It is possible that the differences in worm type and in storage of worms may be a cause of the variation in survival assay results. In the future, these survival assays will be repeated with refrigerated and unrefrigerated worms to see if this accounts for the difference in survival of TN2 between figure 1 and figure 7.

Although we have not obtained consistent results on the virulence and mechanism of 2A and 2B genes, we were able to eliminate other genes in our transposon mutants TN2-TN11 we may have previously thought were important in *B. anthracis* virulence but are likely not. We conclude that loss of only 2B is not likely to affect virulence, but it is still possible that the combination of both the 2A and 2B genes might be necessary for full virulence. We hope that this research demonstrates that invertebrate models of infection can be used to help others narrow down their scope of focus when researching virulence factors of *B. anthracis* or virulence factors of other bacteria in general. With further investigation and characterization, we may be able to come up with targets for novel therapeutic mechanisms to bacterial infections.

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