ANTI-VIRULENCE AS A NEW ANTIBIOTIC CLASS:

SEARCHING FOR NOVEL VIRULENCE FACTORS

IN *B. ANTHRACIS* THROUGH

H_2O_2 SCREENING

by

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ABSTRACT

B. anthracis is a gram-positive, spore-forming bacterial pathogen and the causative agent of the deadly disease, anthrax. This pathogen produces a lethal infection due to the potency of its virulence factors in inflicting harm upon and counteracting host defenses. While anthrax toxin and capsule encoded in the *B. anthracis* plasmids are well-studied, there is minimal research into the over 5,000 chromosomal genes. To identify potential chromosomal virulence factors, a transposon mutant library was created to randomly disrupt genes in the *B. anthracis* Sterne strain's chromosome. This library has been previously used to successfully screen for loss of virulence-associated phenotypes. In our current screen, we examined attenuation of mutants exposed to reactive oxygen species (ROS) in the form of H_2O_2. ROS are released by innate immune response cells and destroy invading pathogens lacking adequate defense mechanisms. While there are some known antioxidant-encoding genes in *B. anthracis*, like the catalase gene, we predict there are others that may influence the bacteria's susceptibility to ROS. To search for additional genes, we screened over 1,300 transposon mutants using H_2O_2 and selected mutants with growth attenuation compared to wild-type *B. anthracis* Sterne. Mutants with increased H_2O_2 susceptibility were further tested to confirm *in-vitro* phenotypes. Ultimately, we want to screen selected mutants in the *G. mellonella* invertebrate infection models to prioritize mutants with both *in-vitro* and *in-vivo* phenotypes. Our goal is to discover novel virulence factors while also developing validated methods and procedures to study *B. anthracis* pathogenesis.

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INTRODUCTION

Bacillus anthracis is a bacterial pathogen responsible for the deadly disease, anthrax. It is a gram-positive, rod-shaped bacteria that can survive for long periods of time in unfavorable conditions due to the resiliency of its endospores. Once their host and environment have been exhausted, the spores enter a state of dormancy, which allows them to survive in environments with minimal resources without having to replicate or metabolize [1]. These spores serve as the infectious agent and they can remain in this dormant state for extended periods of time until they encounter a new host that is suitable for germination into vegetative bacteria. Since *B. anthracis* is primarily a zoonotic disease, this new host is frequently an herbivorous mammal who has encountered the spores in the soil [1]. The most common transmission of anthrax to humans is through contact with these infected animals via consumption of contaminated meat or handling of infected hides [2]. Anthrax can be contracted as a result inhalational, gastrointestinal, injectional, or cutaneous contact with spores. In general, contact with *B. anthracis* endospores via cuts on the skin (cutaneous) or intravenous drug use (injection) will cause necrosis and edema of the infected area [2]. At this stage, the infection is localized and treatable. However, if the infection spreads throughout the body and becomes a systemic infection, the fatality rate increases to 30% [2]. Adversely, if the spores are inhaled, the infection almost never remains localized to the lungs. Once inside their hosts alveoli, the spores are able to enter the bloodstream with ease and progress to the lymph nodes where they can then germinate and spread throughout the body [2]. Since this route of infection allows the bacteria to proliferate so efficiently, inhalation has a fatality rate that approaches 100% [2].

The resiliency of *B. anthracis* spores along with the infection's high fatality rate provide explanations for the use of anthrax as a potential weapon in bioterrorism. In 2001, eleven cases

of inhalational and eleven cases of cutaneous anthrax resulted following letters sent through the U.S. mail containing *B. anthracis* endospores [3]. Of these cases, five people were killed due to inhalational exposure [3]. To combat the impending threat of a repeated attack, the FBI created "Amerithrax" to study both the origin of this attack and novel ways to fight future anthrax infections [3]. These efforts prompted years of investigation into the pathogenesis and virulence factors utilized by *B. anthracis*.

The high lethality of *B. anthracis* is a result of the myriad virulence factors utilized in its attack against its host. *B. anthracis* has two well-studied virulence plasmids, pXO1 and pXO2, that are essential to its virulence. The pXO1 plasmid contains the genetic codes for the three anthrax toxins (edema factor, lethal factor, and a protective antigen) [1]. These toxins target host innate immune cells by hindering the activity of neutrophils and by inducing cell death in macrophages and dendritic cells [1]. By disabling the host's first-line of defense, *B. anthracis* is able to survive, divide, and circulate throughout the host, resulting in a lethal level of toxicity. Additionally, the pXO2 plasmid encodes for the poly-D-glutamic acid capsule, which relies on its negative charge to avoid recognition and phagocytosis by the host's immune system [1]. The value of these factors to the bacterium's pathogenesis can be understood through the *B. anthracis* Sterne strain used in our lab. Unlike the Ames strain, which is fully virulent and contains both plasmids, the Sterne strain lacks the pXO2 plasmid. By removing this single virulent factor, *B. anthracis* loses its advantage over its host's immune system and is safe enough to be classified at a Biosafety Level 2.

Although the critical role these plasmids play in *B. anthracis* virulence is well-known, *B. anthracis* also has over 5000 chromosomal genes whose role in virulence is less well-characterized. Our lab is interested in investigating the extent to which these chromosomal genes

contribute to *B. anthracis* virulence. One way to do this is to test the impact of individual gene disruptions on the bacteria's ability to defend against host immune responses. Using transposon mutagenesis to disrupt regions of the chromosomal genome by inserting a large segment of DNA, Dr. McGillivray created a library of thousands of randomly mutated versions of the *B. anthracis* strains each containing a transposon disruption [4]. This library can then be screened against stressors that mimic host defenses to search for a mutation that results in a loss of virulence-associated phenotypes and consequently, virulence attenuation. The efficacy of this library has been confirmed through the discovery of two novel virulence genes: *clpX* and *yceGH* [4, 5]. *ClpX*, a regulatory subunit of the ClpXP protease, was recognized through hemolytic screening and eventually determined to be a crucial virulence factor responsible for the resistance to host antimicrobial peptides [4]. YceGH was discovered while screening for mutants that could no longer infect *Caenorhabditis elegans*, which served as an invertebrate infection model [5]. Loss of the *yceGH* gene prevented the bacterium from defending itself against reactive oxygen species (ROS) released by the host's innate immune cells [5].

ROS are produced by host mitochondria as a byproduct of metabolism and are used by neutrophils and macrophages in the host's first line of defense against invading microbes [6]. Once these immune cells have phagocytosed the pathogen, high concentrations of ROS react with pathogenic DNA, lipid membranes, iron-sulfur centers, and regulatory proteins to halt the infection [7]. Upon contact with ROS, *B. anthracis* upregulates genes encoding antioxidant enzymes, such as catalase and thioredoxin, as well as proteases and chaperones that construct these enzymes [8]. These proteins scavenge and detoxify surrounding ROS so the bacteria can continue invading its host [8]. Although we know some of the bacterial genes that encode mechanisms for withstanding oxidative stress, we believe there are still more chromosomal

genes that are contributing to the evasion of this host defense strategy. To continue the search for important virulence factors, encoded in *B.anthracis* chromosomal genes, my project focused on screening for mutants that showed an increased susceptibility to hydrogen peroxide (H_2O_2), a type of ROS.

The goal of my project is to identify novel chromosomal genes that help *B. anthracis* defend itself against ROS in its host. Any mutants that exhibited increased virulence attenuation in our *in vitro* screens would then be subjected to an *in vivo* screen using the whole animal invertebrate model of infection, *Galleria mellonella. G. mellonella* is an ideal invertebrate infection model because their size permits direct injection, they can be incubated at 37ºC to optimize bacterial growth, and because their innate immune response has analogs to the mammalian response, including ROS production [9]. By confirming the results of the *in vitro* screen in the *in vivo* infection model, the likelihood of the mutation being involved in virulence increases. The mutants that exhibited a phenotype in both the *in vitro* and *in vivo* screens would be prioritized in future studies.

METHODS

***In vitro* H_2O_2 screen for novel *B. anthracis* mutants**

Previously created *B. anthracis* transposon mutants were extracted from a frozen library, plated on brain and heart infusion (BHI) plates containing 50 μg/mL kanamycin (Kan-50), and grown overnight at 37ºC. Individual colonies of unique transposon mutants were selected the following day and transferred using sterile toothpicks into respective wells in a 96-well plate. Mutants were placed in wells 7-96, while the first wells were reserved for controls and additional testing of the previously isolated mutants, LV1 and LV2 (Hamilton unpublished data). Two

previously characterized mutants, 11F11 (Callaghan and Taylor unpublished data) and 4D5 [10] as our positive controls. One well containing 200 µl BHI was used as a blank negative control that should exhibit no growth. The mutants and controls were added to 200 µl BHI Kan-50 broth in the day 1 96-well plate, which was incubated at 37ºC overnight in static conditions. The following morning, 25 µl from each well in the day 1 plate were transferred to 175 µl of BHI in the day 2 96-well plate and incubated overnight at 37ºC in static conditions. The virulence of each mutant was then challenged against oxidative stress by adding 25 µl of each well from the day 2 plate into the day 3 96-well plate containing 175 µl of a 0.006% H_2O_2 -BHI solution. The day 3 plate was incubated overnight at 37ºC in static conditions. On the last day, the day 3 plate was investigated for attenuation by reading the optical density (OD) of each bacterial culture. An absorbance of 600 nm wavelength on the spectrophotometer was used and the growth of the transposon mutants were compared to the controls.

Large-volume H_2O_2 assay

Previously selected mutants and controls were streaked on BHI Kan-50 plates and grown overnight at 37ºC in static conditions. The following morning, individual mutants were picked into respective 5 mL culture tubes containing 1 mL BHI Kan-50 broth (only BHI was used to grow WT) and incubated to stationary phase overnight at 37ºC in shaking conditions. On day-three, the overnight cultures were diluted in BHI Kan-50. 100 µl of the 1:20 diluted overnight culture was added to a 96-well plate containing 100 µl of a H_2O_2-BHI solution, resulting in a final dilution of 1:40. A gradient of concentrations was tested, ranging from 0.0%-0.036% H_2O_2 in a total volume of 200 µl BHI. This 96-well plate was incubated overnight at 37ºC in static conditions. On day-four, the OD of each well was read by a spectrophotometer using an absorbance of 600 nm wavelength.

This same method was used to examine the efficacy of a transposon mix (Tn mix) as a potential control. I used collections from the transposon mutant library to create Tn mix aliquots containing a random mixture of *B. anthracis* transposon mutants. This mix was grown to stationary phase in 1 mL BHI Kan-50 broth in a 5 mL culture tube and challenged with a H_2O_2 concentration gradient ranging from 0.0%-0.036% and incubated. The OD of this mutant mixture using an absorbance of 600 nm wavelength was compared to that of WT.

G. mellonella **competition assay**

An invertebrate live animal model, *G. mellonella*, was ordered from a bait shop (bestbait.com). The worms were weighed, selecting for those between 180-230 mg, and set aside in four groups of eight. WT, 11F11, and Tn mix cultures were grown overnight in 3 mL of BHI Kan-50 (or BHI for WT) in 5 mL culture tubes. These culture tubes were then incubated at 37ºC in shaking conditions for ~12-15 hours until they reached stationary phase. The following morning, a 1:1 mixture was created by combining 500 µl of the WT culture with 500 µl of either the 11F11 or the Tn mix cultures. These mixtures were then washed and resuspended in 1 mL phosphate buffered saline (PBS) twice. To act as a control for contamination and to ensure a 1:1 ratio, the mixtures were plated on both BHI and BHI Kan-50 plates and incubated overnight at 37ºC in static conditions. The groups of worms were then injected with 10 µl of their respective mixture in the posterior cuticle. A 27-guage needle and automated pump was used to perform the injections. Two groups of the worms (one WT versus 11F11 and one WT versus the Tn mix) were incubated for 6 hours at 37ºC. The other two groups (one WT versus 11F11 and one WT versus the Tn mix), were immediately homogenized for the 0-hour incubation. Before homogenization, the worms were rinsed in 100% pure ethanol and miliQ water for approximately 10 seconds each. Each worm was then added to an impact-resistant screw cap

tube filled with 400 µl of PBS and 250 µl of 1mm ceramic beads. The worms in the tubes were then homogenized through two bead beating cycles of 45 seconds at 4.5 meters per second each. Two control tubes, containing PBS, the 1mm ceramic beads, and either of the 1:1 mixtures were also bead beat to ensure the bacteria were not killed in the beating process. Serial dilutions of the homogenized mixtures were then plated on both BHI and BHI Kan-50 plates and incubated overnight at 30ºC for ~15 hours, or until colonies in the smallest dilution were countable. After the 6-hour incubation was completed for the remaining two groups of worms, they were also rinsed, placed into individual impact-resistant screw cap tubes with PBS and 1 mm ceramic beads, and bead beat. These mixtures were then serially diluted and plated on both BHI and BHI Kan-50 plates, followed by incubation at 30ºC for ~15 hours. The colony-forming units (CFUs) were calculated based on the number of colonies counted on the smallest dilution visible on each pair of plates. The CFUs on the BHI Kan-50 plates were subtracted from the CFUs on the BHI plates to determine the recovery rates of both the WT and Tn mix competition and the WT and 11F11 competition.

RESULTS

In vitro **MIC screen identifies 2 novel transposon mutants**

Our first objective was to screen for novel chromosomal virulence genes in the *B. anthracis* genome by challenging transposon mutants against oxidative stress. Hypothetically, if the mutation occurs in a gene involved in ROS defense, the mutant should exhibit decreased growth compared to WT when grown in this environment. To find these mutants, we needed a concentration of H_2O_2 that did not impair WT growth but was potent enough to inhibit growth of 11F11 and 4D5. We tested the controls against concentrations ranging from 0.0% to 0.2%, and

determined the effective concentration to be 0.006% H_2O_2 in a BHI solution. Once we had an effective H_2O_2 concentration, we were able to screen over 1,300 mutants in search of those unable to survive as well as WT. As Figure 1 depicts, I started by picking individual colonies from the transposon mutant library grown on a BHI Kan-50 plate into individual wells in a 96-well plate containing BHI Kan-50. After overnight incubation, the colonies were transferred to a 96-well plate containing only BHI. On the fourth day, the mutants were challenged with a H_2O_2-BHI solution and incubated overnight. Finally, the OD of the mutants were measured and compared to that of WT, 11F11, and 4D5. Through analyzing the OD of these plates, I identified two mutants with growth attenuation similar to our positive controls, 11F11 and 4D5 which exhibit decreased virulence compared to WT, and labelled them as D3 and B5.

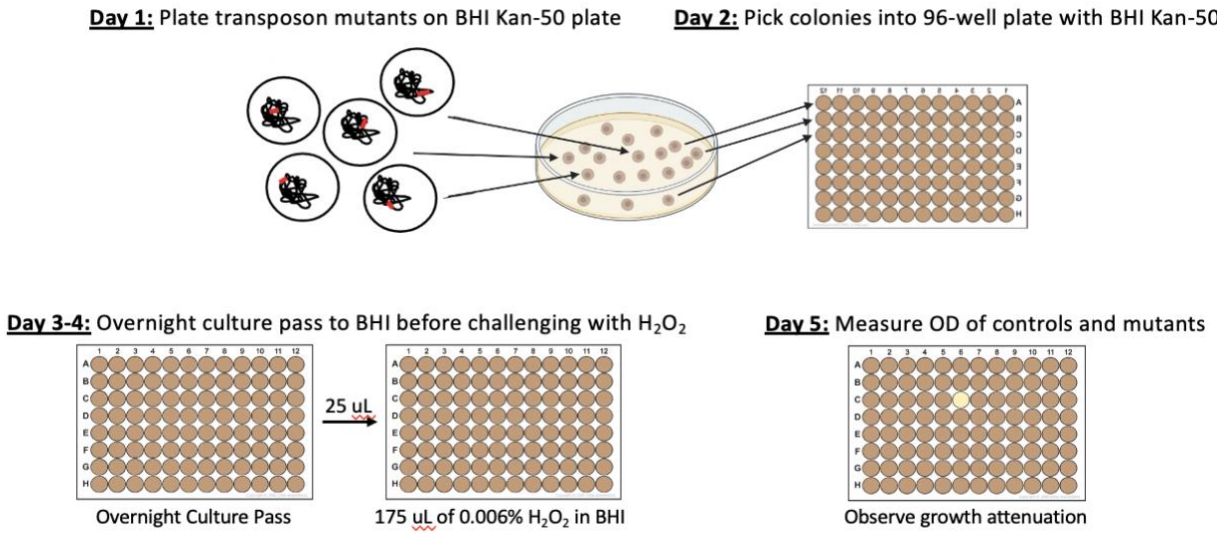

Figure 1. Schematic of *in vitro* H_2O_2 MIC assay. Schematic outlining five-day screening protocol. Controls were in the first seven wells. Transposon mutants were in the remaining 89 wells. The lighter brown well represents the D3 and B5 mutants.

We wanted to then confirm the *in vitro* phenotypes exhibited in these mutants, using a large volume MIC assay using 5 mL culture tubes. Although using 96-well plates allowed many mutants to be screened at one time, they are not ideal for bacterial growth and there can be significant variations in growth from well to well. Therefore, once promising mutants had been extracted, we grew overnight cultures in 5 mL culture tubes. After incubating these cultures overnight, they were diluted 1:40 and added to a 96-well plate containing a 0.016% H_2O_2-BHI solution. This concentration was determined by testing ranges from 0.0% to 0.4% H_2O_2 and choosing the percentage that maintained WT growth, but impaired the growth of 11F11, one of our positive controls. The increased susceptibility to H_2O_2 previously noticed in B5 was not repeated in any further screens. While the D3 mutant would occasionally exhibit growth attenuation similar to our positive control, the results were inconsistent and we concluded its growth was not significantly different from WT (Figure 2). As shown in Table 1, out of the 1,300 mutants screened, none maintained their increased susceptibility to H_2O_2.

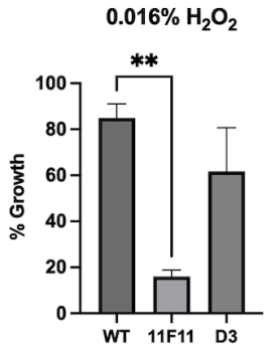

Figure 2. The D3 mutant is not attenuated in H_2O_2. Growth of WT, 11F11, and D3 in 0.016% H_2O_2 in BHI media normalized to growth in 0% H_2O_2 solution. Five individual 96-well plates were screened and a mean +/- standard deviation of these screens is presented. In the panels above, the statistical significance between WT and 11F111, and between WT and Tn mix is designated with **p<.01 by one-way ANOVA followed by Dunnett's multiple comparison *post-hoc* test.

Table 1. Results from initial H_2O_2 MIC screens

Mutants Screened in Initial MIC Assays	Mutants with Increased Attenuation	Mutants with Confirmed H_2O_2 Susceptibility
1,300	2	0

Previously extracted mutants examined in large-volume MIC assays

Prior undergraduates in the lab had extracted five *B. anthracis* mutants (6D10, 35G10, 51D4, 45F1, and 13D4, and LV1) through previous high-throughput H_2O_2 screens similar to mine. I screened these mutants again to confirm their susceptibility to H_2O_2. We assessed the growth of these mutants by growing them overnight in 5 mL culture tubes and then challenging them with 0.016% H_2O_2. The selected mutants were screened using this method five times and the OD of each was compared to the controls. As depicted in Figure 3, none of the mutants showed consistently significant attenuation compared to WT and were therefore not subjected to further testing.

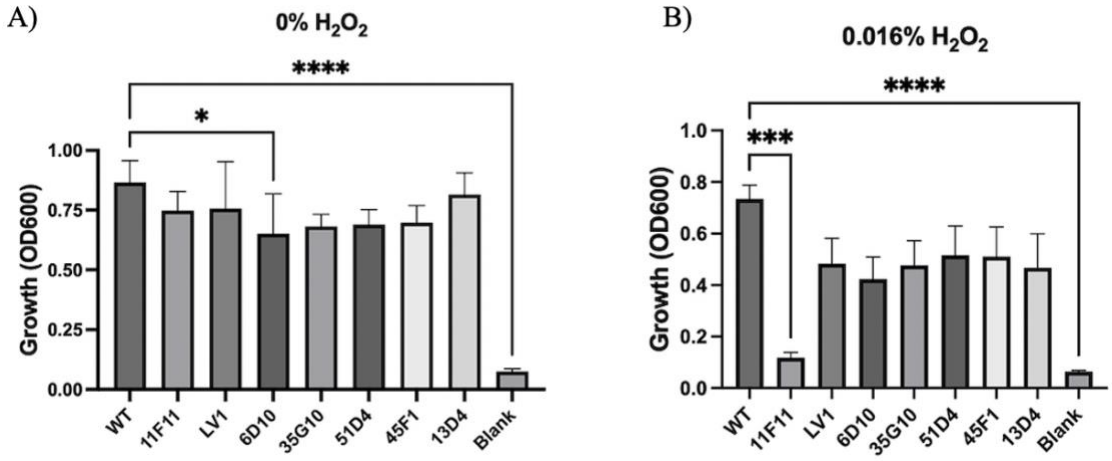

Figure 3. Previously identified mutants were not significantly attenuated in H_2O_2. Growth of WT and previously identified mutants, and a blank control in A) 0% H_2O_2 and B) 0.016% H_2O_2 in BHI media. Five individual 96-well plates were screened and a mean +/- standard deviation of these screens is presented. In the panels above, the statistical significance between WT and 11F111, and between WT and Tn mix is designated with *p<.05, ***p<.001, and ****p<.0001 by one-way ANOVA followed by Dunnett's multiple comparison *post-hoc* test.

Validating *in vitro* and *in vivo* methodologies

My original objective for this study was to identify mutants with increased susceptibility to H_2O_2 using first an *in vitro* and then an *in vivo* assay so I could identify novel genes with a high likelihood of being involved in virulence. However, since the mutants I identified, as well as the ones previously selected, did not maintain a consistent *in vitro* phenotype, I decided to alter my objectives to establish the methodologies behind these screens to assist future research. Previously in our lab, the waxworm, *G. mellonella*, has been used as the invertebrate infection model for screening *B. anthracis* due its size being ideal for injection and its ability to be incubated at 37^{o}C, which optimizes bacterial growth [10]. Furthermore, the innate immune system of *G. mellonella* involves release of ROS, which is similar to the mammalian host defense. These worms have been used to confirm mutant susceptibility to oxidative stress in the past through a competition assay. A schematic of the competition assay is shown in Figure 4. To begin, a 50/50 mix of WT and a transposon (Tn) mutant will be injected into *G. mellonella*. The worms will then be incubated for either 0 hours or 6 hours before they are homogenized and plated on both BHI and BHI Kan-50 plates. Following overnight incubation, the colony-forming units (CFUs) on each plate are counted. The 0-hour worms act as a negative control with growth of WT and Tn mutant being hypothetically equal. This is because the immune system of the waxworms should not have had enough time to eliminate the weakened mutant even if there is a difference in virulence. Since WT lacks the kanamycin-resistance gene, it is unable to grow on the BHI Kan-50 plates. The mutants, however, contain the kanamycin-resistance gene within their transposon insertion, and are able to survive on both BHI and BHI Kan-50 plates. Therefore, the number of CFUs on the BHI plate at 0 hours should be around double that on the BHI Kan-50 plate. This ensures the ratio of WT to Tn mutant is 50/50. In comparison, the 6-hour

incubation should have provided enough time for the ROS released by the waxworms innate immune response to influence survival. If the mutant is attenuated in virulence, the BHI plates should contain significantly more WT colonies and the BHI Kan-50 plates should exhibit little to no CFUs. If the mutant is non-attenuated, the ratio between recovered WT and Tn mutant should remain the same and there would be twice as many colonies on the BHI plate as the BHI-Kan 50.

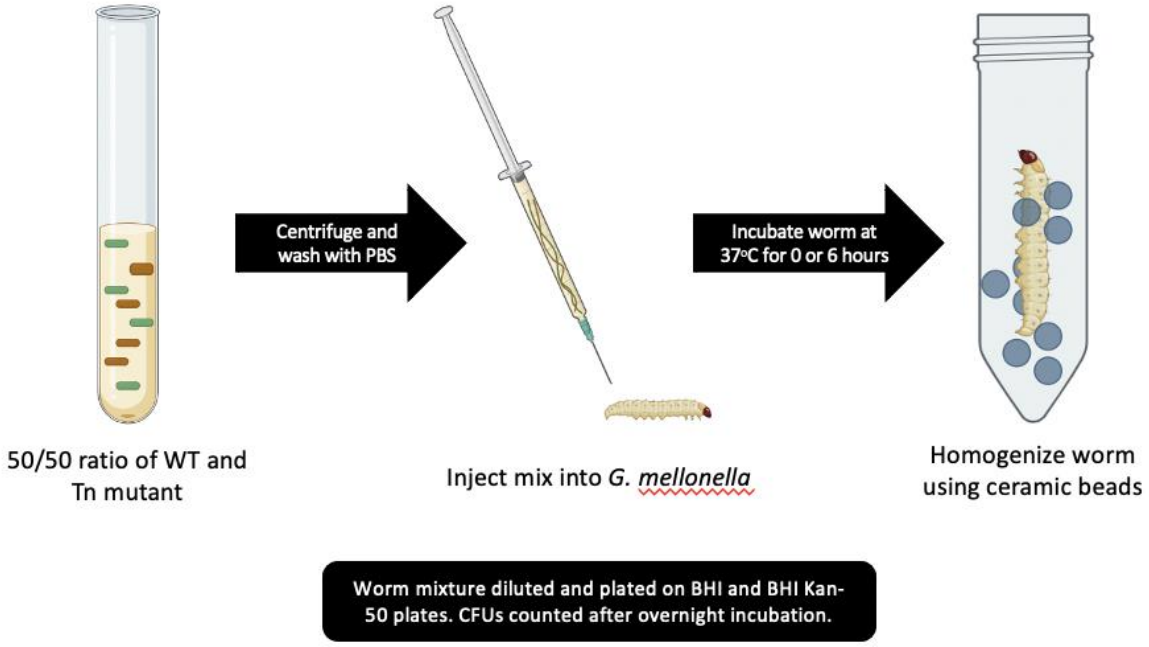

Figure 4: Schematic of *in vivo Galleria mellonella* competition assay. Schematic outlining the *in vivo* competition assay using the *G. mellonella* invertebrate infection model.

Developing a control and negative control

We selected 11F11 as our positive control since the *in vitro* assay results showed that when in contact with H_2O_2, 11F11 is not able to grow as well as WT and because catalase is a known virulence determinant in many pathogens [11]. As a negative control, we decided to create a transposon mix containing multiple different transposon mutants. We reasoned that by

combining hundreds of unique mutants, most should not have a disruption in a gene responsible for ROS resistance. Therefore, this mixture should behave like WT by not exhibiting growth attenuation in an environment of high oxidative stress. To confirm this, we first needed to compared the growth of this transposon mix (Tn mix) to the growth of WT and 11F11 when exposed to H_2O_2 *in vitro*. We investigated this by growing cultures of WT, 11F11, and the Tn mix overnight in 5 mL culture tubes, diluting the cultures 1:20, and challenging them in a 96-well plate against 0.016% H_2O_2. The ODs of the cultures were analyzed the following morning and the results were normalized. As shown in Figure 5, when compared to the growth of WT, there was no significant difference in the growth of the Tn mix. This result provides evidence to support the importance of transposon location on the loss of resistance to environmental stresses.

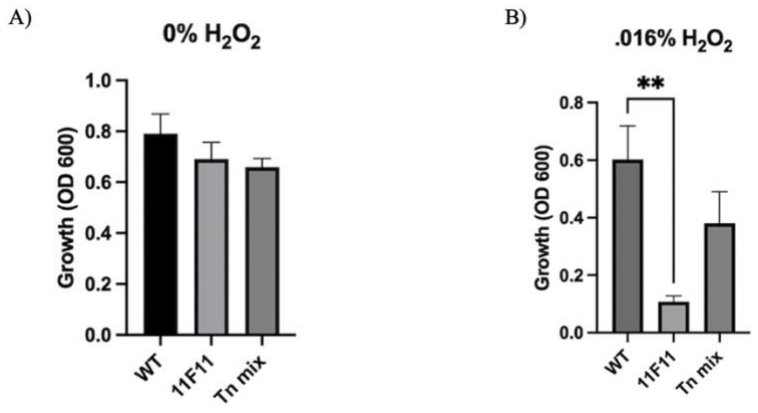

Figure 5. Growth of Tn mix shows no difference compared to WT. Growth of WT, 11F11, and Tn mix in A) 0% H_2O_2 and B) 0.016% H_2O_2 in BHI media. Five individual 96-well plates were screened and a mean +/- standard deviation of these screens is presented. In the panels above, the statistical significance between WT and 11F111, and between WT and Tn mix is designated with **p<.01 by one-way ANOVA followed by Dunnett's multiple comparison *post-hoc* test.

As a final control before this *G. mellonella* competition assay was conducted, we wanted to confirm that the ratios of WT to 11F11 and of WT to Tn mix were 1:1 before injection. We also wanted to confirm the homogenization process using ceramic beads was not killing the

bacteria so we bead beat 400 µl of each 1:1 mixture prior to injection into the waxworms. These results are displayed in Figure 6 and show that the ratios of WT to mutant are approximately 1:1. This ratio was maintained after bead beating with no loss of bacterial survival, indicating that the homogenization of the worms should have no impact on the bacteria.

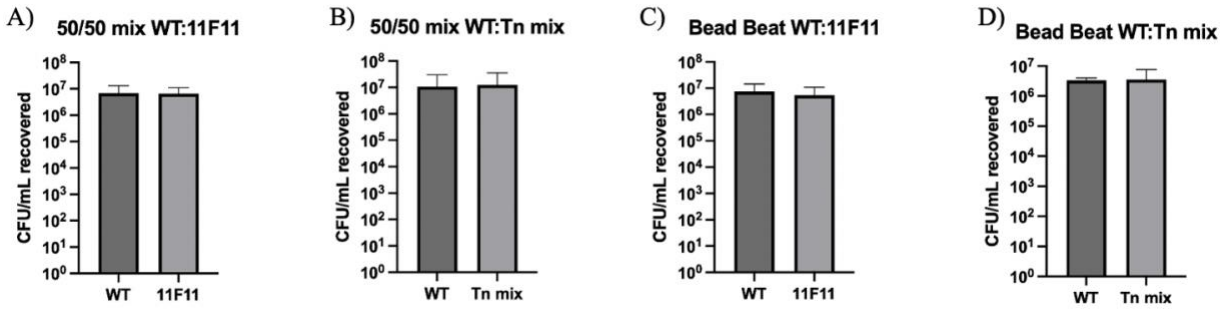

Figure 6. Ratio of WT versus 11F11 and WT versus Tn mix before injection and after bead beating are bout 1:1. A) Growth of WT compared to 11F11 and B) growth of WT compared to Tn mix pre-injection. Six mixtures were created and a mean +/- standard deviation of their growth is presented. C) Growth of WT compared to 11F11 and D) the growth of WT compared to Tn mix after bead beating. Three mixtures were bead beat and a mean +/- standard deviation of their growth is presented.

Our next step was to perform our *in vivo* competition assay. We first tested 11F11, our positive control, that we hypothesized should not survive as well due to the loss of the catalase gene. As expected, there was no statistical difference between the growth of WT and 11F11 at 0 hours, but 11F11 was not able to survive the 6-hour incubation period (Figure 7).

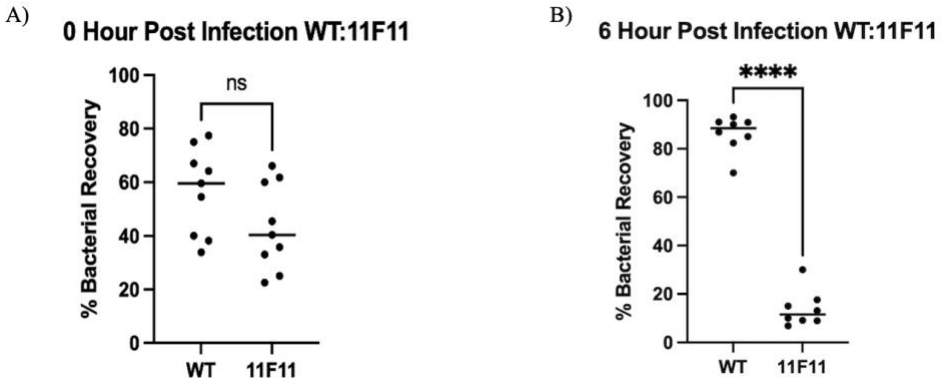

Figure 7: Growth of 11F11 is attenuated compared to WT at 6 hours post infection *in vivo.* Growth of WT and 11F11 after A) 0-hour incubation and B) 6-hour incubation. Two competition assays were performed with the result for each individual worm represented by a dot and the mean is indicated by a horizontal line. In the panels above, the statistical significance between WT and 11F111 is designated with ****p<.0001 or non-significant (ns) by paired t-test.

Although 11F11 worked as a positive control, we still needed to confirm that the observed loss of virulence was caused by the specific disruption of the catalase gene, not merely the presence of the transposon itself. We did this by examining the growth of our negative control (Tn mix) in the competition assay. If this Tn mix was a successful negative control, the mixture would exhibit growth similar to WT at both 0 and 6 hours. However, the results presented in Figure 8 show that at both 0 hours and 6 hours post infection, the growth of the Tn mix is significantly attenuated. These results negate the Tn mix as a reliable negative control. While this negation is due to in part to the mixture being unable to survive the 6-hour incubation period, it is mainly due to its inability to survive the control 0-hour period. Since this mixture of transposon mutants was unable to survive the control incubation period, this Tn mix will not work as an accurate negative control for examining the effect of specific transposon insertions on *B. anthracis* virulence.

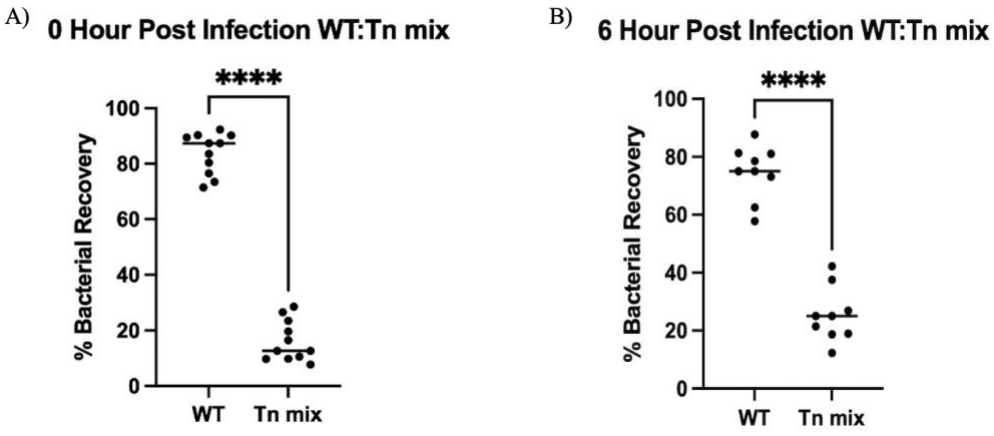

Figure 8: Growth of Tn mix is attenuated at both 0 and 6 hours post infection *in vivo.* Growth of WT and Tn mix after A) 0-hour incubation and B) 6-hour incubation. Two competition assays were performed with the result for each individual worm represented by a dot and the mean is indicated by a horizontal line. The deviation of these screens is presented. In the panels above, the statistical significance between WT and Tn mix is designated with ****p<.0001 by paired t-test.

DISCUSSION

The original goal of our project was to identify novel chromosomal genes involved in *B. anthracis* virulence by challenging transposon mutants against H_2O_2 using MIC screens. Our positive control (11F11) exhibited consistent growth attenuation compared to WT, which validates the efficacy of this screen to identify mutants with transposon insertions that disrupt genes involved in ROS defense. However, 11F11 is not a novel virulence factor, since the gene disrupted is known to encode for the catalase enzyme and this mutant has been previously shown to exhibit increased susceptibility to H_2O_2. Additionally, the other mutants that initially exhibited susceptibility to H_2O_2 that was similar to 11F11 did not replicate this phenotype consistently enough to be significant. The number of these false positives suggests that the concentration of H_2O_2 may have been too high, which would explain why some mutants were unable to survive. Decreasing the H_2O_2 concentration in a future screen would make the screen more sensitive so we would be less likely to pull out false positives and only identify mutants like 11F11 that are truly sensitive to ROS defense. Despite our *in vitro* screen identifying no novel virulence genes, other studies have shown there are still genes besides catalase and thioredoxin in the *B. anthracis* chromosome that are important for ROS-resistance. For example, the *htrA* gene, has been directly linked to H_2O_2 resistance and furthermore acts as a global regulator that influences the expression of thousands of genes when challenged with H_2O_2 [12]. Therefore, we predict that there are still novel virulence genes for ROS resistance in the *B. anthracis* genome even if our screen was unsuccessful in identifying them.

Since no mutants were consistently attenuated in the initial or secondary MIC screens, the goal of our project pivoted to validating our *in vivo G. mellonella* competition assay. We began by confirming that the bead beating process was not killing the bacteria. We also confirmed that

after the 0-hour incubation, growth of our positive control (11F11) and of WT were equal as well as that after the 6-hour incubation, growth of 11F11 was decreased compared to WT. These results confirmed that the presence of a transposon in the *B. anthracis* Sterne strain's genome may cause a loss of virulence in conditions similar to that of the host innate immune response. However, they do not guarantee that this loss of virulence is directly correlated with a specific disruption in a gene responsible for oxidative stress defense. To confirm the importance of transposon location on the ability of our *B. anthracis* transposon mutants to defend themselves against oxidative stress, a mixture of transposon mutants was combined and challenged against H_2O_2. If the location of the transposon was directly correlated with the bacterium's ability to defend itself against ROS, then this mixture should contain enough mutants without a transposon inserted in a ROS resistance gene, that the overall growth should be similar to WT. In our *in vitro* screens, the growth of the Tn mix not statistically less than than that of WT. However, once the *in vivo* competition assay was performed, the growth of the Tn mix was significantly attenuated after both the 0 and 6-hour incubation. Since this mixture is unable to survive in even the 0-hour incubation control, the Tn mix was invalidated as a negative control.

The reason for the growth attenuation exhibited by the Tn mix but not 11F11, especially in the 0-hour control, is unknown, but it is possible that there is an issue with this specific Tn mix. Therefore, in future screens, a different Tn mix (Tn mix 2) should be tested in both an MIC screen and in an *in vivo* competition assay. If this Tn mix 2 exhibits growth attenuation similar to the first Tn mix, the next step will be to complement 11F11. By transforming a plasmid containing the catalase gene back into this mutant, its ability to detoxify ROS should be restored, thus allowing it to survive in conditions of high oxidative stress. This complemented strain

should then act as a negative control by exhibiting growth similar to WT despite the mutant still having a transposon inserted in its genome.

Despite the challenges we faced in developing our *in vitro* and *in vivo* models, it is important to continue developing methods for studying bacterial virulence. Most bacteria employ a multitude of virulence factors that help them successfully surmount their host's immune system. However, many of these factors remain unknown, making the investigation into bacterial pathogenicity a crucial area of research. This is due in part to the general desire to advance science and the simple curiosity into why some bacterial species are able to overcome their host's immune response more easily than others. More significantly, however, learning which virulence factors are important in the bacterium's host infection can provide insight into potential new antibiotic targets. This is known as the anti-virulence strategy of antibiotic development where virulence factors are neutralized through pharmacological targeting thus making it easier of the host immune system to clear the infection [13].

Since the release of the first antibiotic, penicillin, killing invading bacteria by targeting an essential protein has been the conventional strategy used to clear bacterial infections. However, this method has many disadvantages. First, it is relatively unspecific since it targets both pathogens and nonpathogens, which can damage bacteria important to the human microbiome [14]. Second, it puts a survival pressure on the bacteria, which gives the bacteria a need to develop ways to resist antibiotics. For this reason, pathogenic bacteria have been developing resistance to classical antibiotics at a rapid and concerning rate. Alternatively, anti-virulence antibiotics reduce the likelihood of resistance development. This is because targeting virulence factors effectively disarms the bacteria, thus removing the survival pressure placed on the bacteria and allowing the host to clear the infection on its own [14]. Additionally, since virulence

factors are relatively specific to the pathogenic bacteria, targeting these factors would likely not damage the bacteria living symbiotically in the human microbiome [14]. Research into the virulence factors that *B. anthracis* requires to maintain its pathogenicity provides information that can aid in the development of new antibiotics capable of reducing the lethality of an anthrax infection, as well as of bacterial infections in general.

Not only is resistance developing in medical settings, but it is also being transferred between bacterial species in the environment. Numerous strains of non-pathogenic bacteria have evolutionarily developed antibiotic-resistant genes for self-protection that can easily be transferred to pathogenic bacteria through horizontal gene transfer [14]. These resistance genes are also prevalent in both domestic and wild animals, which provides the opportunity for certain strains to begin infecting humans once they acquire the genes that help to evade the human immune responses [14]. As a result, bacteria have been able to develop resistance to every class within a few years after the antibiotic is released. The impact of these antibiotic-resistant bacteria was determined by the Center for Disease Control and Prevention in a study that estimated that in 2013, these infections caused over 23,000 deaths and this number is increasing annually [14].

Compounding problems posed by antibiotic resistance, there are very few new antibiotic targets being identified or new antibiotic classes being developed [14]. Research into suitable targets, including virulence genes, could therefore help expand antibiotic development. This research can only be successful, however, with reliable methods of searching for these new drug targets which includes developing suitable *in vitro* and *in vivo* models. During a time when an onslaught of antibiotic-resistant bacteria that can survive the human immune response is a serious threat, it is paramount that we determine new ways to combat bacterial infections. While my project did not identify any novel virulence factors, it did enhance the methodologies used in

our lab to screen against specific host immune responses to identify the genes that *B. anthracis* uses to cause a fatal infection.

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