DISCOVERING NOVEL GENES IMPORTANT FOR SURVIVAL AGAINST REACTIVE OXYGEN SPECIES IN *BACILLUS ANTHRACIS*

by

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ABSTRACT

Bacillus anthracis is a gram-positive, spore-forming bacterium and the causative agent of the deadly disease anthrax. The *B. anthracis* genome consists of chromosomal genes and the pXO1 and pXO2 plasmids that strongly contribute to the bacteria's deadly nature. While the virulence factors associated with the plasmids have been extensively studied, we believe there are still undiscovered chromosomal genes that may also have important virulence factors. To identify novel chromosomal genes associated with *B. anthracis* virulence, we screened a transposon mutant library of *B. anthracis* Sterne strain for increased sensitivity to reactive oxygen species. Reactive oxygen species such as hydrogen peroxide (H_2O_2) have many functions in mammalian immune defenses and wild type *B. anthracis* is able to subvert this host defense. Sensitivity to reactive oxygen species was tested through *in vitro* H_2O_2 assays and after several rounds of screening seven mutants were confirmed as susceptible. We next tested whether any of these mutants were attenuated *in vivo* using our invertebrate animal model, *Galleria mellonella,* and found several mutants with decreased virulence. We have identified the location the transposon insertion in one of these mutants. High priority for future research will be to determine the sites of disruption of the other promising mutants. This could lead to the discovery of novel *B. anthracis* virulence genes and eventually possible treatment targets for future anthrax outbreaks and attacks.

ACKNOWLEDGEMENTS

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INTRODUCTION

Bacillus anthracis is a gram-positive, rod-shaped soil bacterium. This spore-forming bacterium is the causative agent of anthrax, a deadly disease primarily infecting livestock animals. Human transmission of anthrax occurs by contact with spores via animals or animal products (1). There are multiple types of anthrax: cutaneous, which occurs from abrasions in the skin and accounts for most cases, and gastrointestinal and inhalational, which are rare but often fatal due to systemic spread of the disease (1). Understanding anthrax is important because of its history and possibility of future use as a bioterrorism weapon. In the past, anthrax endospores specifically have been used in bioterrorism because of the potentially high mortality rate of inhalational anthrax and the environmental stability of the bacterial endospore (1).

B. anthracis stands out as a bacterial pathogen in part due to its highly virulent nature. Virulence is a measure of severity of a disease and is important to study because it can help us understand how bacteria cause disease and ultimately lead us to discover new ways to fight pathogens. Pathogens have different virulence factors that work together to invade the host, evade host immune defenses, and cause disease with some pathogens better able to do this than others. *B. anthracis* is known to have two plasmids that contribute significantly to its virulence. The pXO1 plasmid contains exotoxin genes, specifically lethal toxin and edema toxin, which lead to the death of macrophages through oxidative stress (2). The edema toxin interferes with cell signaling to cause production of cyclic AMP, which in turn leads to the production of interleukin-6, inhibition of tumor necrosis factor in monocytes, and inhibition of neutrophil phagocytosis (2). The pXO2 plasmid contains capsule genes that prevent host phagocytosis of the bacterium. Strains lacking the pXO2 plasmid, such as the Sterne strain, have reduced virulence in animal and human hosts and have therefore been used in vaccines (2). The Sterne

strain is also used as a model strain for *B. anthracis* as it can be safely used in a BSL-2 setting (3).

While the plasmid genes are important to study, there are also over 5,000 chromosomal genes in the *B. anthracis* genome (4), some of which are known to contribute to virulence, but many roles of the genes are not yet understood. In 2003, the entire genome of *B. anthracis* was sequenced and found only 2,000 with known function, showing that the function of most *B. anthracis* chromosomal genes is still unknown (4). By comparing the *B. anthracis* genome to sequences of other related bacterial species, *B. anthracis* was found to have chromosomal homologs to several known virulence factors. These homologs include hemolysins, phospholipases, and iron acquisition genes (4). Numerous studies have started to identify the functions of the unknown *B. anthracis* chromosomal genes. Some chromosomal genes have even been characterized as genes specifically associated with virulence. For example, through use of the full genome sequence and various screens, the MntA and HtrA genes have been identified as important *B. anthracis* virulence genes (5, 6). They specifically have been tested and found important to bacterial defense against reactive oxygen species (ROS).

In our lab, we have utilized a transposon mutant library to identify novel *B. anthracis* virulence factors. This library was previously created by exposing *B. anthracis* Sterne strain to a transposon (7), which randomly jumps into different chromosomal sites, thus creating random mutations in the genome. Once created, mutants could be screened for loss of virulence-associated phenotypes, such as the ability to withstand host ROS defenses. This approach successfully identified two chromosomal genes, ClpX and yWLE, that were determined to be important in *B. anthracis* virulence (7,8).

ROS, such as H_2O_2, are a group of reactive chemicals naturally created as a byproduct of cellular respiration. While it has been long known that these can be toxic to cells and cause cell damage, ROS are an important part of the mammalian immune system. ROS, specifically H_2O_2, can diffuse easily to act as intracellular messengers and can also act in cell signaling as second messengers. They can target many different proteins involved in signaling cascades and can also target transcription factors to affect a multitude of cellular processes including cell survival, growth, proliferation, differentiation, senescence, and apoptosis (9). In the immune system, ROS can be created by phagocytic cells to cause direct microorganism killing through an oxidative burst. They also cause lethal DNA damage and can target iron and sulfur containing proteins. In addition to killing pathogens themselves, ROS are associated with other immune defenses including, but not limited to, cytokine release, inflammation, T cell activation, and B cell activation and differentiation (9). Therefore, ROS are a critical aspect of the mammalian immune system that is responsible for defending the host against invading pathogens, like bacteria.

Some bacterial species, including wild-type *B. anthracis*, have evolved defenses to evade host ROS. One of these defenses is catalase, an enzyme that neutralizes ROS into less toxic products. *B. anthracis* also has the ability to inhibit the NADPH oxidase of phagocytic leukocytes, an enzyme the host uses to create ROS to kill phagocytosed bacteria (10). Defense against ROS is obviously important, as can be seen in the decreased survival of *B. anthracis* when MntA and HtrA chromosomal genes are disrupted. Without MntA or HtrA, *B. anthracis* showed increased susceptibility to ROS and decreased virulence (5,6). The importance of ROS in the mammalian immune system can therefore be seen in both the many ROS defenses present in *B. anthracis* as well as attenuated virulence seen when these defenses and associated genes are interrupted.

To identify novel chromosomal genes, I will screen mutants from the previously created transposon library (7) for increased susceptibility to ROS, specifically H_2O_2, in an *in vitro* assay. Once we have narrowed down potential mutants using an *in vitro* screen we will next test them for attenuated virulence in our invertebrate animal model, *Galleria mellonella*. *G. mellonella* has many similarities to the mammalian immune system, including the ability to create ROS (11). While mice have been commonly used as models for the Sterne strain, using a large number of vertebrate animals for this preliminary research is unnecessary and undesirable because of the time and resources associated with vertebrate animals as well as the ethical implications. The larva of the greater wax moth, *G. mellonella*, is an ideal animal model because they are inexpensive and easy to maintain. The larvae can be injected directly and incubated at 37º C, which ensures even distribution of the pathogen and optimal bacteria growth. Use of *G. mellonella* as a model for *B. anthracis* Sterne was recently confirmed through survival and competition assays with *B. anthracis* mutants of previously identified virulence genes (12). Therefore, we believe that any hits confirmed in our *in vivo G. mellonella* infection model are likely to be relevant to virulence in *B. anthracis*.

EXPERIMENTAL PROCEDURES

Primary in vitro H_2O_2 assay

Bacteria were transferred from 96-well frozen stocks using a replica stamper into 96-well round bottom plates containing 180µL of fresh Brain-Heart-Infusion (BHI) media. Plates were wrapped in dry paper towels to reduce evaporation and incubated for 10-16 hours at 37 ºC in shaking conditions. After this incubation, bacteria were transferred using the replica stamper into new 96-well round bottom plates with 180µL of BHI media. They were then incubated for 7-10 hours at 37 ºC shaking. After this, 10µL of the bacterial cultures were transferred to new 96-well plates containing 170 µL of 0.035% H_2O_2 diluted in BHI. The BHI H_2O_2 dilution was made fresh each day to avoid degradation of the H_2O_2. The plates were then wrapped in dry paper towels and incubated for 16-20 hours at 37 ºC shaking. The overall schematic of the assay can be seen in Fig. 1. Bacterial growth was quantified through optical density (OD) at wavelength 600 nm. Each frozen 96-well plate of mutants was tested 5-6 times.

Secondary in vitro H_2O_2 assay

Attenuated mutants were tested again in a secondary H_2O_2 screen. A new frozen 96-well plate was created with only the mutants found to be attenuated in the primary screen. A wild-type *B. anthracis* control, 4D5 control, and BHI only control were also included. Mutants from frozen stocks were streaked out on BHI agar plates and grown overnight in 37 ºC static conditions. A single colony from each mutant was transferred to the 96-well plate containing 180µL of BHI and grown overnight in 37 ºC aerobic conditions. Bacteria were then transferred to a new 96-well plate with 80% glycerol in a 3:1 ratio of bacteria to glycerol and frozen. This

frozen 96-well plate was then tested in the same protocol as the primary *in vitro* H_2O_2 screen. Additional H_2O_2 concentrations of 0.07% and 0.14% were also tested.

In vivo G. mellonella assay

Experimental procedures for the *in vivo G. mellonella* assay were based on methods from Malmquist et al. (12). Mutants found to be attenuated in the *in vitro* H_2O_2 assays were further tested in the *G. mellonella* wax worm model. *G. mellonella* worms were purchased from Rainbow Mealworms (www.rainbowmealworms.net) and stored at 4 ºC to cause worm torpidity. Only worms weighing 190-220mg were used in the assay. Depending on the condition and weights of the worms received, 10-15 worms were used in each condition. Every trial consisted of a 1x Phosphate Buffer Saline (PBS) control, wild-type *B. anthracis* control, 4D5 mutant control, and 3-4 mutants being tested. Mutants from frozen stocks were streaked out on BHI agar plates and grown overnight in 37 ºC static conditions. A single colony from each mutant was transferred to culture tubes with 1-2mL of BHI and grown overnight. 150µL of overnight cultures were transferred to a new culture tube with 3mL of BHI and grown in 37 ºC shaking conditions to log phase (OD=400) for approximately two hours. Once the bacteria had reached log phase growth, 1000µL was transferred to a 1.5mL microcentrifuge tube and centrifuged for 5 minutes at the highest speed. The bacteria were washed and resuspended in 1000µL 1x PBS. The bacteria were then diluted 1:2 in fresh 1x PBS. An automated pump (New Era Pump Systems NE-500, Farmingdale, NY, USA) and 27-gauge needle were used to inject 10µL of the 1:2 bacteria dilution into the posterior cuticle of each worm (hour 0). After injection, worms were incubated at 37 ºC and the number of surviving worms was recorded at 24, 48, and 72 hours after injection. Immediately after injecting the worms, each 1:2 bacterial dilution was further diluted

and plated to determine the exact colony forming units (CFUs) that were injected into each condition of worms. From the 1:2 bacteria PBS solution, 1:100, 1:1000, and 1:10000 dilutions were created and 25µL of each dilution was plated on BHI agar plates. After 24 hours of incubation in 30 ºC static conditions, colony-forming units (CFU's) were counted.

Identification of Transposon Insertion

Isolation of Genomic DNA

Genomic DNA was isolated from selected mutants using phenol-chloroform extraction. Mutants from frozen stocks were streaked out on BHI agar plates and grown overnight in 37 ºC under static conditions. A single colony from each mutant was transferred to culture tubes with 10 mL of BHI and grown overnight. The 10mL overnight culture was centrifuged at 3750rpm for 10 minutes. The bacterial pellet was resuspended in 655µL of protoplast buffer (8mL of 25% sucrose, 200µL of 0.5M Tris-HCl, 100µL 1M $MgCl_2$, 5µL 100% Triton X, and distilled water to 10 mL) in a 1.5mL microcentrifuge tube. 200µL of 100 mg/mL lysozyme was added and solution was incubated for 60 minutes at 37ºC. Next 25µL of 20% SDS and 10µL of 10mg/mL RNAse A was added and the solution was incubated for 60 minutes at 37 ºC. Then 10µL of 20 mg/mL proteinase K was added and the solution was incubated for 60 minutes at 50-55 ºC. An equal volume of phenol-chloroform, 1mL, was added and hand-mixed through tube-inversion. The microcentrifuge tube was then centrifuged for 5 minutes at 10,000rpm and afterwards the upper aqueous layer was extracted to a new 1.5mL microcentrifuge tube. The phenol-chloroform extraction was repeated, centrifuged, and upper aqueous layer transferred to the same 1.5mL microcentrifuge tube from the previous reaction. 1/10 volume of 3M sodium acetate and 0.6 volumes of isopropanol were added and hand-mixed gently until the DNA precipitated out. DNA

was then centrifuged for 5 minutes at 10,000rpm and washed with 500µL of 70% ethanol. The DNA pellet was dried for 5-10 minutes at room temperature and resuspended in 100µL of water. Absorbance was measured at OD=600.

Y-linker Creation, Bacterial Digest and Ligation

Experimental procedures for the Y-linker method were adapted from methods based on Kwon et al. (13). The Y-linker was created by annealing two partially complementary primers called linker 1 (5′-TTTCTGCTCGAATTCAAGCTTCTAACGATGTACGGGGACACATG-3′) and linker 2 (5′-TGTCCCGTACATCGTTAGAACTACTCGTACCATCCT-3′). First, 18µL of linker 2 (100µM) with PNK (2µL enzyme, 4µL 10x ligase buffer, 16µL water) in a 1.5mL microcentrifuge tube at 37ºC for 1 hour. PNK was then heat denatured at 65ºC for 35 minutes. 18µL of linker 1 (100µM) was added to a final volume of 58µL. The mixture was heated at 95ºC for 5 minutes and then allowed to cool to room temperature. Isolated DNA from each mutant of interest was digested with the enzyme NlaIII (NEB cutsmart buffer) for 3 hours at 37ºC. 200ng of DNA was ligated to 5µL of Y-linker with 1µL T4 DNA ligase and 2µL 10x ligase buffer to a final volume of 20µL. The ligation reaction solution was incubated overnight at room temperature. The reaction was diluted to a final volume of 200µL and heated at 65ºC for 10 minutes.

PCR and Sequencing

2µL of ligated DNA was amplified through PCR with 2µL of the Y-linker primer (5′-CTGCTCGAATTCAAGCTTCT-3′), 2µL of the transposon specific primer Himar 1-2 long (5′-GGGAATCATTTGAAGGTTGGTACT-3′), 2µL 10x buffer, 2µL dNTPs, and 1µL Taq polymerase to a final volume of 20µL. The PCR product was then purified with the Promega Wizard SV Genomic DNA purification system and sent for sequencing using the Himar 1-4

primer (5′ -TATGCATTTAATACTAGCGAC-3′). Sequenced DNA was then BLASTed to analyze which genes were interrupted by the transposon. After the correct sequencing results were achieved, a confirmation primer downstream of the transposon insertion site was used in colony PCR. The mutant from frozen stocks was streaked out on a BHI agar plate and grown overnight in 37 ºC. A single colony was transferred to a PCR tube with 10µL of water and microwaved for one minute. 1µL of DNA and water solution was amplified through PCR with 2µL of the 1F11 specific confirmation primer (5′ -GTATATTCGTGAATCCCGCTGC-3'), 2µL of the transposon specific primer (Himar 1-2 long), 2µL 10x buffer, 2µL dNTPs, and 1µL Taq polymerase to a final volume of 20µL.

RESULTS

In vitro assays

Our first objective was to determine the screening conditions that would allow us to reliably identify mutants attenuated for survival in H_2O_2. We first had to determine conditions where all mutants would grow approximately equal in the absence of H_2O_2. The different conditions included the type of 96-well plates used, adding dry paper towels around the plates to reduce evaporation, using a replica well plater rather than individual pipettes to transfer the bacteria, and allowing two rounds of bacterial growth in fresh media before testing in H_2O_2 (as can be seen in Fig. 1). We next had to determine the H_2O_2 concentration that would allow wild-type *B. anthracis* Sterne to grow but not mutants with a growth defect in the presence of H_2O_2. An H_2O_2 concentration of 0.035% in the primary *in vitro* screen was found to be the best because it allowed both wild-type to survive and some mutants to show attenuation.

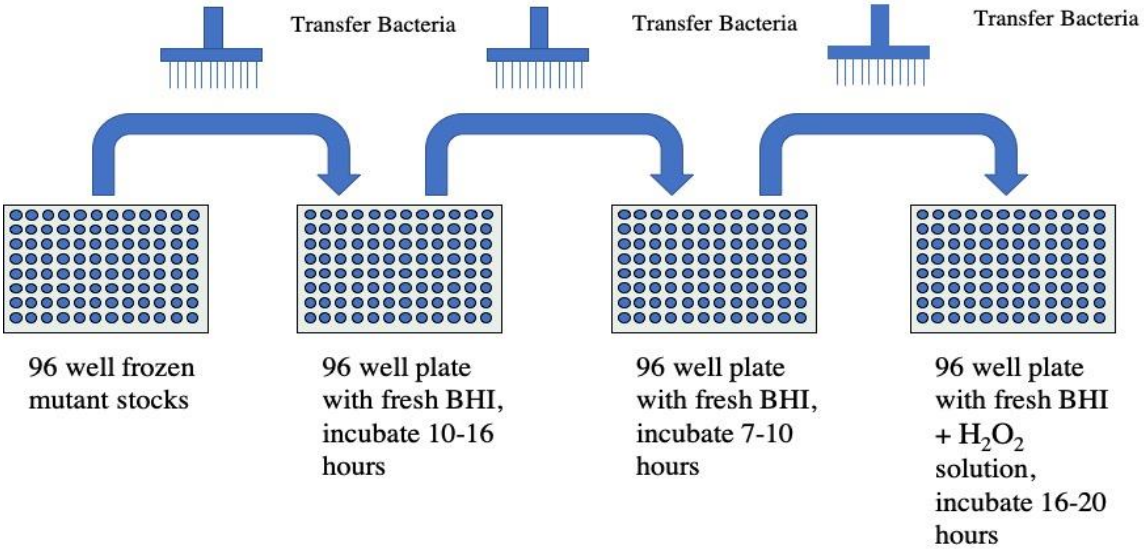

Figure 1 H_2O_2 screen schematic. Bacteria were transferred from frozen stocks stored in 96 well plates to a new 96 well plate with 180µL fresh BHI, incubated shaking at 37 ºC for 10-16 hours, transferred again to a new 96 well plate with 180µL fresh BHI, incubated shaking at 37 ºC for 7-10 hours, transferred to a 96 well plate with H_2O_2 BHI solution and incubated shaking at 37º for 16-20 hours before measured at OD=600.

Total Plates Screened	Total Mutants Screened	Total Mutants Selected from Primary Screen	% Hits from Primary Screen
21	1,953	40	2.048%

Table 1 Summary of primary screen results.

Once assay conditions were established it was determined that a 2% hit rate in our *in vitro* screen was achieved. Wild-type and unaffected mutants that grew normally typically had an OD reading of 2.0-3.5 whereas attenuated mutants had OD readings less than 1.0. Each time a plate was screened, it was noted whether a specific mutant was attenuated. Because results were not 100% consistent between screenings, every plate was analyzed separately multiple times (see Table 2 for details). For any attenuated mutant, we then calculated how many times that particular mutant was attenuated after every assay performed. Mutants with attenuated survival that repeated > 67% were counted as a "hit" (summarized in Table 2). Many of these mutants were tested in a secondary H_2O_2 screen to confirm their phenotype but additional secondary screens still need to be conducted.

In the secondary *in vitro* screen, we re-tested 17 of the 40 mutants identified in the primary screen. These 17 mutants were randomly selected and the rest of the mutants will be tested in the future. Table 2 shows which mutants were tested (highlighted in blue) and which still need to be tested in another secondary screen. The secondary screen was performed with a range of H_2O_2 concentrations, as can be seen by the results in Fig. 2. Controls for this screen included wild-type *B. anthracis* and *B. anthracis* mutant 4D5, a mutant previously discovered to have attenuated virulence in both the invertebrate *G. mellonella* as well as mammalian models of infection (12, 14). In addition to attenuated virulence, 4D5 is also associated with decreased defense to oxidative stress (14).

Plates Screened	Number of Times Screened	Mutants Selected	% Times Mutant Repeated as Attenuated in Primary *in vitro* screen	Tested in Secondary Screen
1	4	1F11 1F10 1C10 1C11	75% 67% 67% 67%	Yes No No No
2	6	2B11 2D12	80% 83.4%	No Yes
3	5	3D2 3D3 3E12 3F12	67% 67% 83.4% 83.4%	No No No Yes
4	4	4E12	75%	No
5	4	5D6 5F12	75% 75%	No No
6-7	4-5	None		
8	3	8A4	67%	Yes
9	4	9E5 9F2	75% 75%	Yes Yes
10	4	none		
11	4	11F11	100%	Yes
12	3	12B3 12D4 12D6	67% 67% 67%	No Yes No
13	3	13C3 13D4	67% 67%	No Yes
14	4	14C5 14D3 14D4 14D6 14D7 14E3	75% 100% 100% 75% 75% 75%	No Yes Yes No No Yes
15	4	none		
16	3	16D9 16E3 16E8 16F9	67% 100% 67% 67%	No Yes Yes No
17	3	17B7 17D8 17E8	67% 67% 67%	No No No
18	3	18F5	100%	Yes
22	3	22E3 22E7	67% 67%	No No
23	3	23F4 23F8	67% 67%	Yes Yes
24	2	None		

Table 2 Primary screen results for each 96-well plate screened.

We established screening conditions where wild-type would survive in the H_2O_2 and 4D5 would show decreased growth. The approximately equal growth of all bacterial mutants in 0% H_2O_2 (Fig. 2A) shows all mutants are able to grow successfully under the experimental conditions and in the absence of H_2O_2. Growth in 0.035% and 0.07% H_2O_2 (Fig. 2B and C) show 11F11 is more highly attenuated than all of the other mutants and is the only one to show significantly decreased growth in the lowest H_2O_2 concentration of 0.035%. With an increase in H_2O_2 (Fig. 2C and D), more mutants can be seen as attenuated but their decrease in growth is not as extreme as with 11F11. Further testing of these mutants should be done, specifically 23F4 and 12D4, which looked promising. Results are summarized in Table 3. In total, we found 7 mutants that were consistently attenuated in H_2O_2 (8A4, 9F2, 9E5, 11F11, 14D3, 13D4, and 14E3).

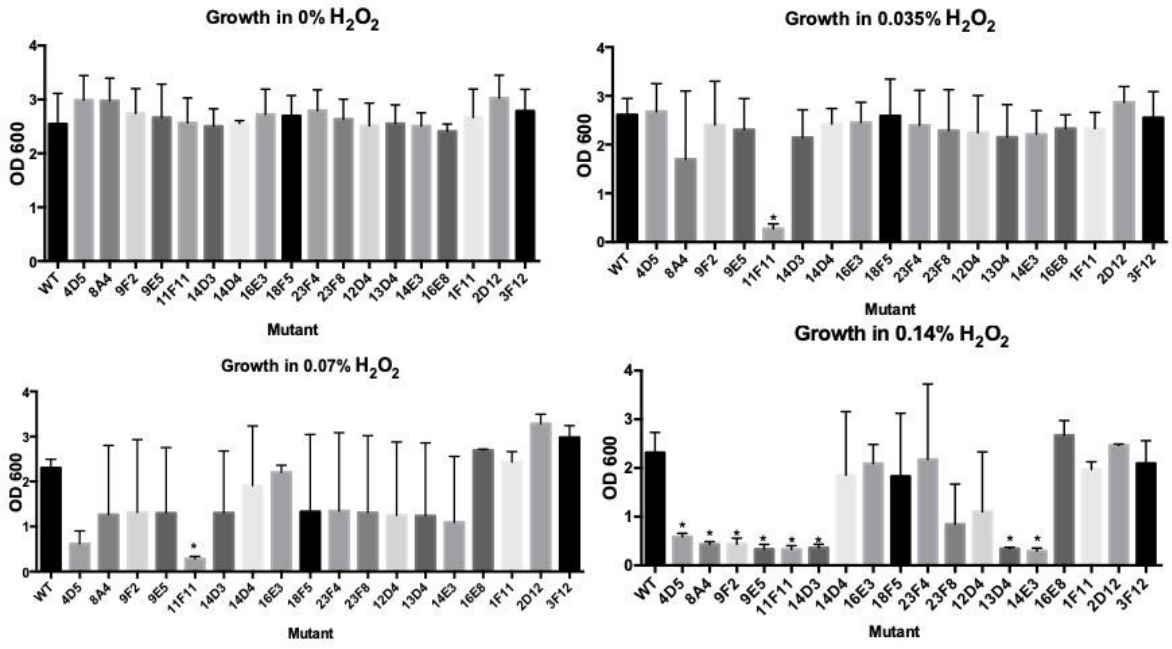

Figure 2 Growth of *B. anthracis* mutants in H_2O_2. Wild-type (WT) and Transposon Mutant *B. anthracis* growth in 0% (A), 0.035% (B), 0.07% (C), and O,14% (D) H_2O_2 BHI solution, data is presented as mean +/- SD from combined results of three experiments. Indicates statistical significance by one-way ANOVA multiple comparisons test, $p<0.05$.

These 7 mutants were divided between Lauren Callaghan and I for further analysis. This included testing these mutants in our *in vivo G. mellonella* infection model and then determining the site of transposon disruption for any mutant with an *in vivo* phonotype.

Mutant	% Survival Relative to WT in *in vitro* Secondary Screen
1F11	85.28%
2D12	106.56%
3F12	90.47%
8A4	18.56%
9E5	14.26%
9F2	18.88%
11F11	14.07%
12D4	47.76%
13D4	14.77%
14D3	15.56%
14D4	80.10%
14E3	12.56%
16E3	90.31%
16E8	86.62%
18F5	78.89%
23F4	93.93%
23F8	36.45%

Table 3 Summary of secondary H_2O_2 screens

G. mellonella assays

I tested four of the seven attenuated H_2O_2 mutants in our *in vivo G. mellonella* screen: 8A4, 9F2, 9E5 and 1F11. While 1F11 was not attenuated in our H_2O_2 model, it was included in an initial *G. mellonella* assay before the secondary screen results were finalized. In addition to the four transposon mutants, all *G. mellonella* trials consisted of a 1x Phosphate Buffer Saline (PBS) control to show injection did not affect worm survival, wild-type *B. anthracis* Sterne, which can successfully infect *G. mellonella* (12) and the 4D5 mutant, which had been previously shown to have decreased virulence in *G. mellonella* (12). Worms were divided into groups of 10-

15 worms weighing 190-220mg. This weight range was important to ensure worms were receiving an equal magnitude of bacteria upon injection.

Figure 3 *G. mellonella* larvae infected with *B. anthracis* Sterne. Larvae that are living are yellow and able to move. Dead larvae have turned black. Black spots start to appear before the larvae dies.

Worms were injected in the posterior cuticle with bacteria grown to log phase (OD=400). After injection, worms were incubated at 37 ºC and the number of surviving worms was recorded at 24, 48, and 72 hours after injection. Survival was assessed through appearance of the worm. When *G. mellonella* dies, the worm becomes black whereas living worms are able to move and have a yellow color, as can be seen in Fig. 3. Statistical significance was assessed through a log rank test in comparison to wild-type survival. Larvae injected with wild-type had 16.67% survival rate. Interestingly, although 1Fll did not have a phenotype in our H_2O_2 assay, this mutant is much less virulent than wild-type with 86.67% *G. mellonella* survival rate. Other mutants tested were also found less virulent than wild-type: 8A4 had a 75.86% worm survival rate, 9F2 had an 80.0% worm survival rate, and 9E5 83.33% worm survival rate. Therefore, we find that all four mutants tested had significantly decreased virulence compared to wild-type, as can be seen in Fig. 4.

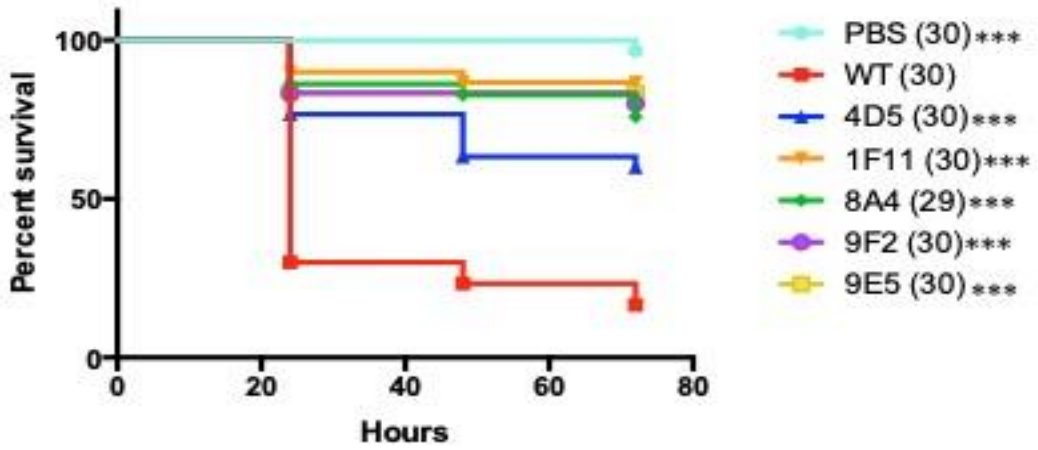

Figure 4 *G. mellonlla* larvae infected with *B. anthracis* mutants survival assay. Number of surviving worms was recorded at 24, 48, and 72 hours. Results of three experiments, total larvae indicated in parentheses. *Indicates statistically different survival than WT, $p<0.001$ by log rank test.

Identification of Transposon Insertion

Mutants found to be attenuated in both the *in vitro* and *in vivo* screens were selected for identification of the site of transposon insertion using the Y-linker method (13).

Briefly, genomic DNA was isolated, and the DNA was then digested with NlaIII, which recognizes a 4 bp sequence that will cut frequently in the genome. The fragmented DNA was then ligated to the Y-linker, formed from two partially complementary oligonucleotides with compatible NlaIII sticky ends. PCR was then performed using a primer specific to the transposon sequence and a primer specific to the Y-linker. Successful amplification would thus include both transposon and genomic sequenced that could be BLASTed for homology (see the schematic of the Y-linker method Fig. 5).

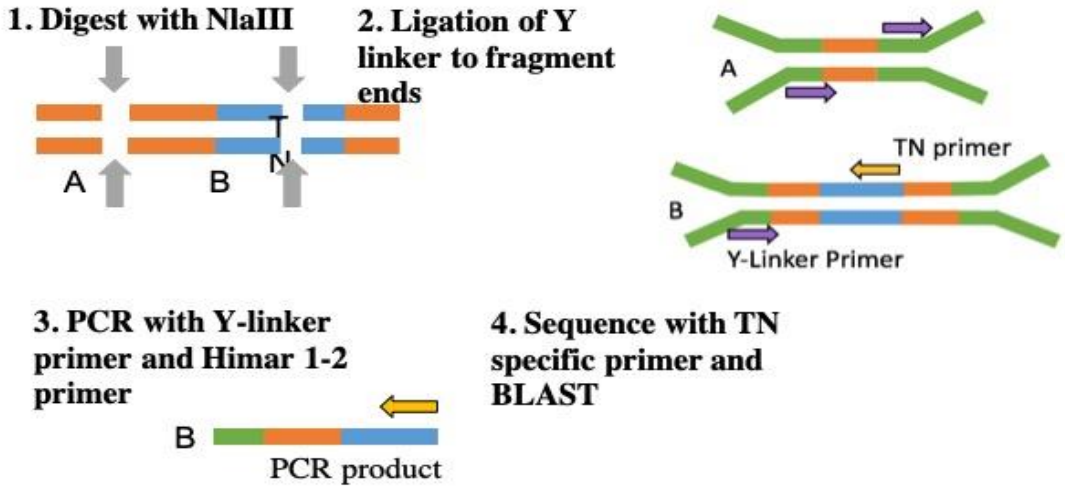

Figure 5 Schematic of the Y-Linker method. Transposon (TN) mutants of interest were digested with NlaIII, ligated with a Y-linker. DNA was amplified through PCR and products were sequenced and BLASTed. (TN = transposon)

Fig. 6 shows a successful PCR with clear bands for each mutant including the control mutant 22B3. This method was successfully performed on 22B3 in earlier studies and is a control to demonstrate that our Y-linker method was done correctly.

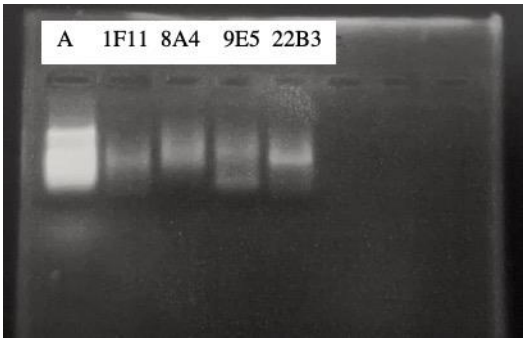

Figure 6 Initial PCR results. PCR amplification of digested genomic DNA after ligation to Y-linker run using the Y-linker primer and Himar 1-2 long primer. A is a ladder.

Unfortunately, this initial PCR was difficult to repeat and we spent a large amount of time optimizing PCR conditions, including running gradients of annealing temperatures to determine the optimal annealing temperature. These gradient PCRs can be seen in Fig. 7.

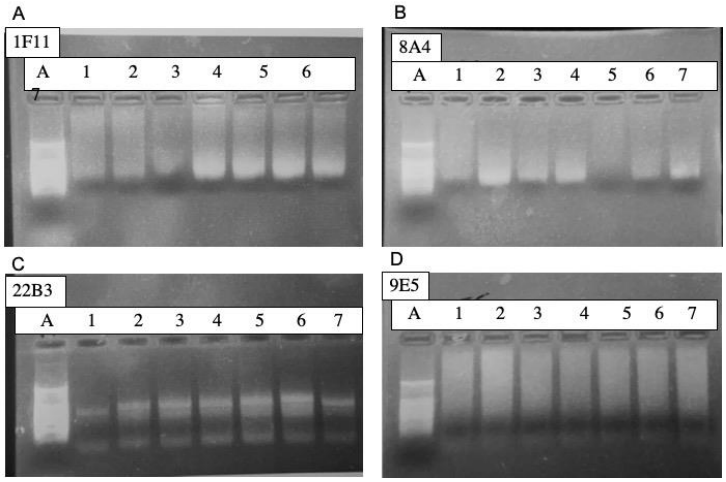

Figure 7 Gradient PCR results. PCR amplification of digested genomic DNA after ligation to Y-linker using the Y-linker primer and Himar 1-2 long primer. A is a ladder. Wells 1-7 represent the same PCR reaction with increasing annealing temperatures (54º-62º).

DNA isolated from PCR results from 1F11, 22B3, 8A4, and 9E5 (Fig. 7) was sequenced with the Himar 1-4 primer put successful sequencing results were only obtained for 1Fll (Fig. 7A) and the control 22B3 (Fig. 7C). Genomic sequence from 1F11 can be seen in Fig. 8. The first highlighted section in blue is the transposon sequence and the second highlighted sequence in green is the complementary sequence to the 1F11 confirmation primer. PCR amplification using the 1F11 confirmation primer and Y-linker primer would only occur if the transposon were inserted in that position. The results from the PCR with 1F11 and its confirmation primer can be seen in Fig. 9.

CTTAAAGGACGAAACGGTGCATTATCAGCCAACCTGTTATCTTCTTTTATACGTCCCAATCTAATCCGT
CATTACGTTCATATTTTTATATATGAGGAGGTGACGAACATAACTCATATCATTGATTACCAAGGTATT
CAGCCAATAAATAAAACGGATGCAACGACTTTTACAATTCCCAACTCCCCAAACAAAGCAATTCTAGTA
AATATTGAATTAAAAATTCCACTAAAATATTCACGCAATAATAGAATAGAATTAATTACCACAATTGGT
TTTAAAAGTGTAACAAATAGATCACAATTATTTGTTAGAATTTTCCGTAACGACATTGATATTTTTAAC
ACACAAGTAAGTATAGGCTCCACAGATTATAAACAATATTCAGTTGAAACATTTCAAACTATTGATAAA
AATGTAAGCAGCGGGATTCACGAATATACTTTAACTGTGGAAAATCTAACAAGTGATGCAAGTGCACAT
GTCATTGGGTCCCCCTCTCTTTTTAGTGGGTTATCAATTGGGACAAGTTTATAAATTCCTATTAAGCAA
ACCCTTCCTCAAAATATTATATTTTGAGAAAAAGGATGACGGATTAT

Figure 8 1F11 sequence results. Isolated Genomic DNA sequenced with transposon-specific Himar 1-4 primer. First blue highlighted section is transposon sequence and second green highlighted sequence is complementary sequence to the 1F11 confirmation primer.

In this PCR, 1F11 does have a bright, clear band. Wild-type *B. anthracis* DNA (WT) served as our negative control. This lane has some faded banding, which is most likely due to non-specific priming (Fig. 9). I intended to repeat this PCR with a higher annealing temperature but unfortunately was unable to do so with the campus closure. Nonetheless, these results indicate that our sequencing was successful, and the transposon inserted in a hypothetical protein shown in Fig. 10, as determined from BLASTing the sequence. The genes surrounding this disruption are also shown.

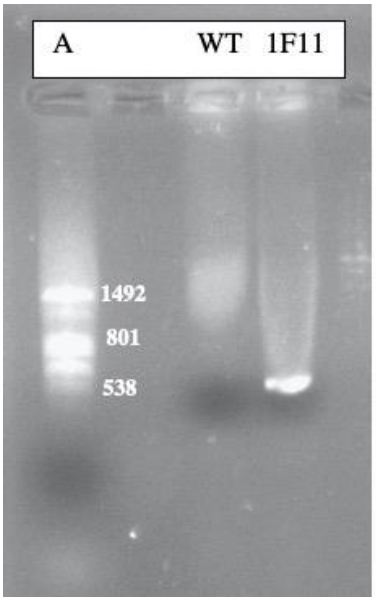

Figure 9 Confirmation of the site of the transposon insertion in 1F11. PCR amplification of WT and 1Fll genomic DNA using 1F11 confirmation primer and Himar 1-2 long primer. A is a ladder.

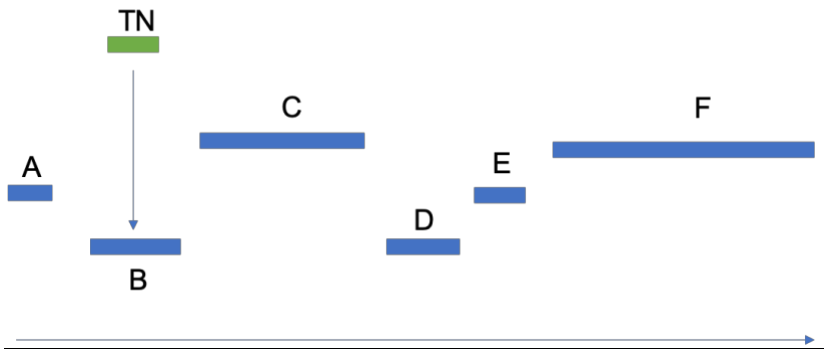

Figure 10 Genes disrupted by 1F11 transposon insertion. Genes in and around the site of transposon insertion (arrow) found in gene B. Genes are annotated as: A: hypothetical protein, B: hypothetical protein, C: MFS transporter, D: hypothetical protein, E: lipoprotein, F: LL-diaminopimelate aminotransferase

DISCUSSION

Our goal was to use a combination of *in vitro* and *in vivo* screens to identify relevant *B. anthracis* mutants with potentially novel virulence genes. In our primary *in vitro* screen, we screened 1,935 mutants for susceptibility to H_2O_2 and identified 40 potentially attenuated mutants, which is a 2.048% hit rate. This hit-rate is consistent with previous *in vitro* screens conducted in our lab (7, 8) and therefore we believe our *in vitro* conditions were well-optimized. From there, we screened 17 mutants in a secondary *in vitro* screen and identified 7 attenuated mutants. Time constraints prevented us from screening the other 23 mutants, which will be tested by future students in the lab. Additionally, conditions need to continue to be perfected to reduce the large error bars seen with some of the mutants in our secondary screen. Conducting assays in a larger volume could help improve consistency as the 200µl limit of 96-well plates prevents good aeration and can cause more variations in growth. Next, we tested 8 mutants in our *in vivo G. mellonella* model. I focused on 4 mutants and found that all 4 were attenuated *in vivo*. The remaining 4 mutants were tested by Lauren Callaghan as part of her research project and she found 2 of the 4 to be attenuated in the *G. mellonella* model. In total, we found 75% of the mutants tested *in vivo* had a phenotype. While finding a phenotype in *G. mellonella* was most important, the primary and secondary *in vitro* screens were helpful in narrowing down the almost 2,000 mutants in a timely manner to ones that should be tested in the longer and more labor intensive *in vivo* screen. Combining both these screens allowed us to successfully narrow down a large number of mutants to a smaller number of highly relevant ones that were feasible to pursue.

While all 4 of my transposon mutants were attenuated in the *G. mellonella* data, I was only able to determine the site of transposon disruption for 1F11. As can be seen in Fig. 10, I found that 1F11 disrupts a hypothetical protein. Because of close-by genes in the same coding

direction, there is reason to believe this hypothetical protein is part of an operon. Upstream of the insertion site is gene encoding a hypothetical proteins. This is unlikely to be disrupted by the transposon since the regulatory element regulating its expression remains undisturbed. Downstream proteins include an MFS transporter and a LL-diamniopimelate aminotransferase (DapL). Expression of these genes are likely to be decreased due to the transposon insertion as this large of an insertion would disrupt regulatory elements and prevent efficient transcription of these genes. MFS transporters belong to a large family of membrane transporters and are involved in multiple cellular functions, including solute uniport, solute/cation symport, solute/cation antiport and/or solute/solute antiport (15). DapL is an enzyme that transfers nitrogenous groups in a recently discovered variation of the DAP/lysine (DapL) pathway. DAP is an important amino acid utilized in cross-linking of peptidoglycan to create the bacterial cell wall. Because of its function, bacterial mutants with an interrupted DapL pathway would likely be more susceptible to antibiotics that target the cell wall. This DapL pathway was found in pathogenic bacteria from the genera *Chlamydia*, *Leptospira*, and *Treponema*, providing an opportunity for development of narrow spectrum antibiotics. (16).

It is interesting to note that 1F11 did not have a phenotype in the *in vitro* screen but did in the *in vivo* screen. I speculate that, because 1F11 was found to disrupt a protein important for cell wall synthesis, the bacteria may not make as effective of a cell well. In the *in vitro* screen with H_2O_2, this may not have been a harmful enough mutation to cause death. In the *in vivo* screen, however, *G. mellonella* could have additional defenses other than ROS that are specifically designed to target the bacterial cell wall and thus in that environment, the mutation is much more detrimental to the bacteria. As mentioned earlier, this mutant was included in *G. mellonella* screens before the secondary *in vitro* screen was completed and thus this mutant was found

almost by accident. There could be other mutants, like 1F11, we did not find attenuated in H_2O_2 that could be attenuated in the wax worm model. To find these mutants, additional screens could be designed that mimic other aspects of the mammalian immune system.

In the future, we will need to create an independent mutant knocking out the gene 1F11 was found to disrupt. Attenuation with this independent mutant would show that these genes are associated with virulence, not an unknown mutation somewhere else in the genome that is actually contributing to the phenotype. If this confirms our mutant, further testing could be done such as *in vitro* assays looking at antimicrobials that target the cell wall. Ultimately, testing in mammalian models would be ideal as this would show whether the mutation disrupts genes important for virulence in the mammalian immune system.

There are also 5 other mutants with an *in vivo* phenotype that should also be further studied. The transposon disruption site of one of these mutants, 13D4, was successfully found by Lauren Callaghan. It has a disruption in an operon containing a Na+/Ala symporter protein and the Dps2 protein, which is associated with a stress response that has some links to hydrogen peroxide resistance (17). Our independent attempts have generated two highly interesting mutants. Neither of them has been directly linked to virulence before so potentially implicating them in disease pathogenesis is a novel finding and warrants further study. It is important to identify virulence factors for several reasons. Virulence factors are what the bacterium use to invade and establish infection in a host. Pharmacologically inhibiting them could potentially hinder the bacteria from causing infection and make it easier for the host immune system to clear the pathogen. For this reason, targeting virulence factors is a new strategy in antibiotic development (18). This is especially important as antibiotic resistance continues to increase so rapidly. We believe future studies either continuing our screening system or following up on the

mutants we have identified will provide us a better understanding of *B. anthracis* virulence, and since bacterial pathogens often share virulence mechanisms, a better understanding of bacterial pathogenesis in general.

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