DISCOVERING NOVEL GENES THAT ALLOW *BACILLUS ANTHRACIS* TO SURVIVE HOST DEFENSES

by

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DISCOVERING NOVEL GENES THAT ALLOW *BACILLUS ANTHRACIS* TO SURVIVE HOST DEFENSES

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ABSTRACT

Bacillus anthracis is a bacterium that causes the deadly disease anthrax and has been used in bioterrorism. We are looking to investigate what genes within the chromosomal DNA contribute to the virulence of *Bacillus anthracis*. In this study, we screened a transposon library of *B. anthracis* 'knock-out' mutants for susceptibility to reactive oxygen species used by the immune system. A broad *in vitro* hydrogen peroxide screen was performed on 1,953 transposon mutants, and after several rounds of *in vitro* screening, 40 mutants were identified as consistently attenuated in the presence of hydrogen peroxide. Four of these mutants were then tested in the invertebrate model, *Galleria mellonella*, to assess virulence in an animal model. Mutants with phenotypes that repeated in both assays were prioritized for characterization. The location of the transposon insertion in one of the mutants was successfully identified. Identifying these novel genes contributing to the bacterium's virulence will provide a better understanding of *B. anthracis* pathogenesis and may provide potential targets for combatting anthrax.

INTRODUCTION

Bacillus anthracis is a gram-positive, rod shaped bacteria that is the causative agent of the disease anthrax. The bacterium is soil-dwelling and is endemic throughout much of the world from northern Canada[1] to the Steppe region in Kazakhstan[2]. *Bacillus anthracis* is also a spore forming bacteria and therefore will, upon environmental pressures, form spores for survival. Sporulation allows the bacteria to remain dormant in a vegetative state, requiring few, if any, nutrients. Spores are also extremely resistant to many harmful agents such as ultraviolet light, antibiotics, and heat. As conditions return to ones favorable for growth and reproduction, the spores will germinate back into a viable bacterium able to infect a host [3]. *Bacillus anthracis* is a human pathogen, but human-to-human transmission has not been documented. Human infection primarily occurs due to the handling of infected livestock or animal products such as wool or meat, as *B. anthracis* is primarily a zoonotic pathogen among herbivorous animals [4]. Anthrax is often a highly treatable disease, but current treatment is not ideal as antimicrobial therapy is often long, with antibiotics prescribed for approximately 60 days [3]. A vaccine has also been developed, but it requires multiple rounds of injections and annual boosters to maintain its effectiveness [5].

The most common form of infection among human hosts is subcutaneous anthrax due to spores or bacteria entering a cut or scrape. A distinguishing sign of this infection is the appearance of an ulcer with a black eschar, surrounded by swelling [6]. If left untreated, most patients will fully recover within two to six weeks, though 20% of infections may become septic and lethal. However, with full access to antibiotics and healthcare, subcutaneous infections

often have very favorable outcomes. The survival rate is approximately 90-100% when antibiotics are given in conjunction with antibodies that neutralize the anthrax toxins. In addition, because of the distinguishing presentation of the black eschar, early diagnosis of subcutaneous anthrax is relatively unproblematic, which contributes to the high survival rate. These subcutaneous cases make up approximately 90% of the reported cases of anthrax [3].

Other sources of documented anthrax infection are due to injection and inhalation. These cases of anthrax infection are far more critical with much less favorable outcomes. Extensive data does not exist on the mortality rates due to the rarity of these infection pathways, but estimates originally placed the mortality rates of ingestion and inhalation anthrax infection at 95%. More recently, the mortality rate was only 45% among the 11 patients during the bioterrorism attack in 2004, but this higher survival rate may be due to early knowledge that the infection was due to anthrax as stated in one of the bioterrorist's letters [3]. Without explicit knowledge of infection by *Bacillus anthracis*, diagnosis of the infection in a clinical setting often proves much more difficult as inhalation anthrax presents with non-specific flu-like symptoms. Because of this, the infection often will reach systemic proportions prior to diagnosis, leading to the patient's death even with interventions [3]. The combination of durable spores, which can serve as viable infectious agents for decades, and the confounding generic, flu-like symptoms that can lead to misdiagnosis and higher mortality rates, can make *Bacillus anthracis* a highly potent bioterrorist threat [7].

The high pathogenicity of *Bacillus anthracis* is due to a number of virulence genes encoded in its genome, which includes around 5000 chromosomal genes and two plasmids,

pXO1 and pXO2. Once a *B. anthracis* spore has entered a host, germination will occur, and the bacteria will rapidly divide. The bacteria will also produce the capsule, encoded on the pXO2 plasmid, to prevent detection and phagocytosis by the immune system. The Sterne strain of *B. anthracis* lacks this defensive plasmid and, therefore, is nonpathogenic in humans [3]. This lack of a capsule allows for the study of the bacterium and its virulence without the need for highly protective equipment. The pXO1 plasmid produces the two anthrax toxins, lethal toxin and edema toxin, which interrupt neutrophil signaling and promote apoptosis for dendritic and macrophage cells [3]. With the immune system disarmed against the bacteria, *Bacillus anthracis* will disseminate throughout the body via the lymphatic system. Once a systemic infection is established, toxin concentrations will continue to increase until hemorrhaging, shock, and multi-organ failure kill the host [8]. The anthrax toxins and capsule are integral for the pathogenicity of *Bacillus anthracis*, and mutants missing either plasmid are attenuated for virulence [6].

More recently the research focus has also shifted to include the importance of chromosomal genes to virulence and survival. This part of the genome of *B. anthracis* is less studied but is likely to also have key roles in virulence. For example, *Bacillus subtilis* and *Bacillus cereus* are two model organisms for the *Bacillus* family of bacteria, and through the comparison of chromosomal genes and proteins, both have revealed virulence homologues to genes found in *B. anthracis* [9, 10]. One example is *ccpA*, a chromosomal regulator of the carbon metabolism in *Bacillus subtilis*, and mutants with this gene, or other homologous genes, interrupted display virulence attenuation [11, 12]. Throughout our study we will use *B. anthracis*

mutant 4D5 which has a disruption of the petrobactin biosynthesis operon [13, 14]. This operon is important for iron acquisition, resistance to reactive oxygen species, and virulence in animal models [14, 15]. These are just a few examples of known chromosomal genes that are essential to *B. anthracis*'s virulence, and with approximately 5000 chromosomal genes, this leaves the opportunity that unstudied chromosomal genes may still be discovered that also contribute to this potent virulence.

A common method of studying chromosomal genes is the production of knock-out mutants via transposon mutagenesis. Transposons are often encoded on a separate plasmid that, once introduced into a bacterium, will jump from the plasmid into the chromosomal DNA at a random spot in a process called transposition [16]. If the transposon inserts itself within a gene or regulatory element, it will disrupt the gene, usually rendering it inactive. This allows the phenotypic difference between the mutant and wild-type bacteria to be observed in order to deduce the functionality of the gene interrupted. By using this technique, our lab has previously created a library of *B. anthracis* transposon mutants, each theoretically with a different interrupted gene. This library has been previously screened for hemolytic activity to identify the *clpX* gene, which is essential for defense against antimicrobial peptides and bacterial virulence [17], as well as the yceGH gene, which contributes to virulence through defense against reactive oxygen species (ROS) [18]. The technique can be applied to investigate many different aspects of host-pathogen interaction in hopes to again reveal a novel gene contributing to the ability of *B. anthracis* to evade the immune system and establish an infection.

This project aims to discover novel chromosomal genes that are important for defense against reactive oxygen species (ROS), specifically hydrogen peroxide, which are a critical aspect of innate immune defense. In most living organisms, ROS combine with metals such as iron and undergo a reaction called the Fenton's reaction that produces highly toxic hydroxyl radical which can damage DNA and can ultimately cause cell death [19]. Many bacteria are susceptible to the production of this toxic hydroxyl radical, which is one reason many immune cells, like phagocytes, use ROS in the immune system's defense against pathogens. However, wild-type *Bacillus anthracis* demonstrates a strong resistance to ROS and is able to infect and reside in immune cells like macrophages [20]. It has previously been determined that the bacterium does have existing antioxidative genes that are essential for its virulence and survival in its host, including an endogenous nitric oxide synthase, which is used to activate catalase, a protein that degrades ROS. Once activated, catalase is able to degrade not only the endogenous ROS nitric oxide but also exogenous ROS produced by the host's immune system that would otherwise kill the bacterium [21]. This example is just one of many known techniques that different bacteria utilize to defend against ROS. Multiple other pathways are present in other species such as the use of different superoxide dismutase enzymes in many ROS-resistant pathogens such as *E. coli*, *Salmonella*, and *A. baumannii* [22, 23]. This class of antioxidant enzymes is also present in multidrug-resistant strains of bacteria, and upon inhibition of the enzyme, survival and pathogenicity of the bacteria is greatly decreased [23]. This indicates the importance for bacteria to possess genes to combat hosts' immune systems in order to survive,

and when characterized, may also then serve as possible targets to stop the bacteria's ability to establish an infection.

We will use our library of *B. anthracis* Sterne transposon mutants to screen approximately 2000 mutants for increased susceptibility to hydrogen peroxide using a high throughput *in vitro* assay. Once we identify promising hits *in vitro*, we will next screen these hits in the invertebrate infection model, *Galleria mellonella*. This model uses the larval form of *G. mellonella* that can be directly injected with a bacterial pathogen [17]. It has not only been validated as a valid *in vivo* infection model for *B. anthracis* [13], but also contains less constraints than a traditional vertebrate model [17]. This worm model also has been shown to share many of the conserved innate immune system components, and therefore will allow us to evaluate mutants' virulence in an animal model [24]. We believe this two-part screening method, using both *in vitro* and *in vivo* screens, will be key to our success. Use of the *in vitro* screen first will allow us the ability to investigate a large number of mutants, and the *in vivo* model will help ensure that the hits identified are likely to be relevant for virulence in an animal model. Mutants that demonstrate attenuation in both the *in vitro* and *in vivo* assays will be prioritized for follow-up and characterization.

METHODS

***In vitro* Hydrogen Peroxide Screening Protocol.** Frozen stocks of the different *B. anthracis* Sterne transposon mutants created previously [17] were used to start the cultures for screening. They were first grown in 96-well round-bottom plate, labeled 'stamp plate,' with 180 µL of brain and

heart infusion media (BHI). This stamp plate was incubated shaking at 37°C for ~12-18 hours. For best results throughout this protocol, the 96-well round-bottom plates were wrapped in paper towels to prevent friction in the shaking incubator and media evaporation. After incubation, a new 96-well round-bottom plate was labeled 'culture plate,' filled with 180 µL of BHI, and each well was inoculated with the corresponding mutant from the stamp plate. The culture plate was incubated shaking at 37°C for ~8-9.5 hours. A 0.035% hydrogen peroxide BHI media solution was then prepared, and 180 µL of H_2O_2 media was pipetted into each well of a new 96-well round-bottom plate labeled 'screen plate.' Using the 'culture plate,' 15 µL of each mutant was pipetted into the corresponding well on the 'screen plate'. To ensure even inoculation, each well on the 'culture plate' was pipetted up and down a few times prior to taking the 15 µL to ensure full resuspension of the culture. This screen plate was incubated shaking at 37°C for ~15-20 hours. The optical density (OD) of each well of the 96-well screen plate was read using the absorbance at 600 nm wavelength.

***In vivo Galleria mellonella* Survival Protocol.** *G. mellonella* worms were obtained from an online bait shop (www.rainbowmealworms.net) and placed in the fridge to decrease movement for accurate weighing. The worms were weighed, and those with a mass of 190-220 mg were selected and recorded. Ten to fifteen worms were used for each mutant. Worms were placed back in the fridge until the trial. Overnight cultures were grown in BHI and incubated shaking at 37°C. For each *B. anthracis* Sterne strain, 100 µL of the overnight culture was used to inoculate 3 mL of BHI and incubated shaking at 37°C until log phase was reached with an optical density

of approximately 0.4. A 1-mL aliquot of the log phase culture was then pelleted, and the supernatant fluid removed. The pellet was washed with phosphate buffered saline (PBS) and resuspended in PBS. A 1:2 dilution was created, and 10 µL of the 1:2 dilution was injected into the corresponding worms. The worms were incubated at 37°C and checked at 24-hour intervals for 3 days to record death. The corresponding 1:2 dilution was also used to complete a 1:10 serial dilution, and the 1:200, 1:2000, and 1:20,000 dilutions were plated to determine the approximate cfu of culture injected into the worms. These plates were incubated overnight at 30°C and cfu calculated.

Y-Linker PCR. This protocol was adapted from Kwon and Ricke [25]. Primers used are listed in Table 1. Linker 1 and 2 primers were diluted to 100 µM. A 2-µL aliquot of PNK was used to phosphorylate 18 µL of Linker 2 in T4 ligase buffer for one hour at 37°C. It was then placed in 65°C for 20 minutes to heat denature PNK. An 18-µL aliquot of Linker 1 was added and heated to 95 °C for 5 min. It was then allowed to slowly cool to room temperature. Bacterial genomic DNA was extracted using phenol/chloroform technique, and 5 µg of the DNA was digested with *NlaIII* (NEB cutsmart buffer) for at least 3 hours at 37 °C. Ligation of the DNA to the Y-linker was performed by adding approximately 200 ng of digested DNA to 5 µL of Y-linker with 1 µL of T4 DNA ligase (NEB) and 2 µL of 10x ligase buffer. This was incubated overnight at room temperature. The mixture was diluted to 200 µL and heat denatured at 65 °C for 10 min. An aliquot of 2-5 µL of ligated DNA was used as the template in the PCR with the Himar 1-2 Long and Y-linker primers to amplify the fragment of bacterial DNA containing part of the

transposon sequence. The amplified DNA product was purified following the manufacturer's protocol (Promega). The purified product was then sequenced using primer Himar 1-4 and BLASTed against the wild-type *B. anthracis* genome to determine transposon location.

Confirmation PCR. One colony of the mutant 13D4 was mixed with 10 µL of water in a PCR tube and microwaved for 1 minute. A 1-µL aliquot of this DNA was then used as the template for the PCR. The primers used are listed in Table 1. Himar 1-2 Long was used as the transposon specific primer, and 13D4 primer was used as the *B. anthracis* mutant 13D4 specific primer.

Table 1. List of primers used in Y-linker and Confirmation PCR protocol.

Primer Name	Primer Sequence
Linker 1	5' - TTTCTGCTCGAATTCAAGCTTCTAACGATGTACGGGGACACATG - 3'
Linker 2	5' - TGTCCCCGTACATCGTTAGAACTACTCGTACCATCCACAT - 3'
Y-linker Primer	5' - CTGCTCGAATTCAAGCTTCT - 3'
Himar 1-2 Long	5' - GGGAATCATTTGAAGGTTGGTACT - 3'
Himar 1-4	5' - TATGCATTTAATACTAGCGAC - 3'
13D4 Primer	5' - GCTACCCATACCTGCTTCTG - 3'

RESULTS

High-throughput screening in 96-well plates identifies mutants of interest.

The first goal of this study was to determine a protocol to screen approximately 2000 mutants from the transposon library. These mutants were individually arrayed as frozen aliquots in twenty 96-well plates. With a large number of mutants to screen, an efficient method was needed that yielded consistent results for the reliable selection of phenotypically interesting mutants. Mutant 4D5 was selected as a positive control, as this mutant has been previously shown to demonstrate attenuation in the presence of reactive oxygen species [13]. After multiple trials, we determined that between growing the initial inoculation from the frozen stock and the final H_2O_2 assay plate, an additional culture plate intermediate was needed for consistent results. Because our initial inoculation was performed using frozen stocks, the amount of bacteria inoculated varied between mutants, and therefore, affected the consistency of results in the assay. To correct this, the initial plate, deemed the "stamp plate," was inoculated from the original frozen library plate and grown in fresh media overnight. This plate was used to inoculate the intermediate "culture plate", which was grown for about 8 hours and then used to inoculate the H_2O_2 assay plate. By creating the intermediate culture plate, the amount of bacteria was more standardized between mutants, yielding more consistent and reliable results. The concentration of hydrogen peroxide that attenuated the positive control, 4D5, but did not attenuate the negative control, WT, was found to be 0.035% H_2O_2. Once determined, the *in vitro* screening protocol was applied to the 1953 mutants screened. Out of

the 1953 mutants, 40 mutants were indicated as mutants of interest and represent 2.10% of the overall screened mutants (Table 2).

Table 2. Summary of Screenings.

Total Plates Screened	Total Mutants Screened	Total Mutants Selected from Primary Screen	% of Mutants Eligible for Secondary Screening
21	1,953	40	2.10%

Taylor Kelly, another undergraduate research student, and I independently screened each plate twice. After screening a group of 4 plates, we would compare results qualitatively, if growth was visually seen in the wells, and quantitatively, through the OD readings. Both qualitative and quantitative values were considered to ensure that the reading of OD matched the amount of growth seen. If the results were consistent between our trials, we would select mutants of interest based on attenuated growth compared to wild-type. If not, the plates would be rescreened, as necessary. Through this process, each plate was independently screened a total of 4-7 times to ensure the most reliable results. Because mutant growth was not always consistent, as mutants may grow in one trial plate but not in others, we agreed that the designation of 'mutants of interest' would be given to those that demonstrated high rates of attenuated growth and grew in 33% or less of the screening trials (Table 3). From these 40 mutants of interest, 17 were randomly chosen to be re-tested in our secondary screens. This is indicated in the fourth column of Table 3. A future student, or students, will test the remaining mutants in follow-up studies.

Table 3. Summary of Primary Screen Results by Plate.

Plate #	Number of Times Plate Screened	Mutants Selected	% of Times Growth Seen in H_2O_2 Assay	Tested in Secondary Screen
1	4	1F11	25%	Yes
		1F10	33%	No
		1C10	33%	No
		1C11	33%	No
2	5	2B11	20%	Yes
		2D12	16.6%	Yes
3	6	3D2	33%	No
		3D3	33%	No
		3E12	16.6%	No
		3F12	16.6%	Yes
4	4	4E12	25%	No
5	4	5D6	25%	No
		5F12	25%	No
6	4	none		
7	4	none		
8	3	8A4	33%	Yes
9	4	9E5	25%	Yes
		9F2	25%	Yes
10	4	none		
11	4	11F11	0%	Yes
12	3	12B3	33%	No
		12D4	33%	Yes
		12D6	33%	No
13	3	13C3	33%	No
		13D4	33%	Yes
14	4	14C5	25%	No
		14D3	0%	Yes
		14D4	0%	Yes
		14D6	25%	No
		14D7	25%	No
		14E3	25%	Yes
15	4	none		

16	3	16D9	33%	No
		16E3	0%	Yes
		16E8	33%	Yes
		16F9	33%	No
17	3	17B7	33%	No
		17D8	33%	No
		17E8	33%	No
18	3	18F5	0%	Yes
22	3	22E3	33%	No
		22E7	33%	No
23	3	23F4	33%	Yes
		23F8	33%	Yes
24	2	None		

Our secondary screen was performed to ensure that the mutants were truly attenuated in the presence of hydrogen peroxide. Because the primary screening was performed over the course of approximately 6 months, the secondary screening would allow all the mutants of interest to be repeated using the same conditions in one testing environment to ensure the best results. The results of the secondary screening can be seen in Figure 1. The mutants were tested in 0%, 0.035%, 0.07%, and 0.14% hydrogen peroxide conditions following the *in vitro* screening protocol. A concentration of 0% H_2O_2 was used to demonstrate that all cultures were viable and grew equally well in the BHI media (Figure 1, Panel A). At a concentration of 0.035% H_2O_2, only mutant 11F11 was attenuated, demonstrating that this mutant should be prioritized for the *in vivo* trials due to its high susceptibility to hydrogen peroxide. However, our positive control, mutant 4D5, was not attenuated in growth even though it is known to be susceptible to

hydrogen peroxide [13]. Since a known peroxide-susceptible mutant, mutant 4D5, was able to grow and did not show attenuated growth, this means that at this level of hydrogen peroxide potential mutants of interest may be missed. This indicates that the level of screening is too stringent as hydrogen peroxide levels are too low, and therefore, the concentration of hydrogen peroxide in the media should be raised. At a concentration of 0.07% H_2O_2, the results were varied with mutants attenuated in some trials and not in others. This indicated that the concentration was on the edge of the desired concentration that would produce consistent attenuation of the mutants of interest. At a concentration of 0.14% H_2O_2, consistent attenuation of 4D5 was seen.

Additionally, mutants 8A4, 9F2, 9E5, 11F11, 14D3, 13D4, and 14E3 displayed statistically significant attenuation ($p<0.05$) when compared to wild-type growth in the same environment. Other mutants such as 14D4, 18F5, 23F4, 23F8, and 12D4 were varied in the results and were at times attenuated. These had large error bars and, therefore, were not statistically significant when compared to wild-type. These mutants should be further investigated to determine if they have a phenotype of attenuation in the presence of oxygen reactive species and whether they are mutants of interest. The mutants 16E3,16E8, 1F11, 2D12, and 3F12 consistently did not show attenuation in the secondary screen and were comparable to the wild-type growth in the same conditions. After conducting the primary and secondary screening of the mutant library, we found a total of 7 mutants with consistent attenuated growth in H_2O_2 relative to wild-type. These mutants were divided among Taylor Kelly and I for *in vivo* testing and further characterization. I received mutants 11F11, 13D4, 14D3, 14E3.

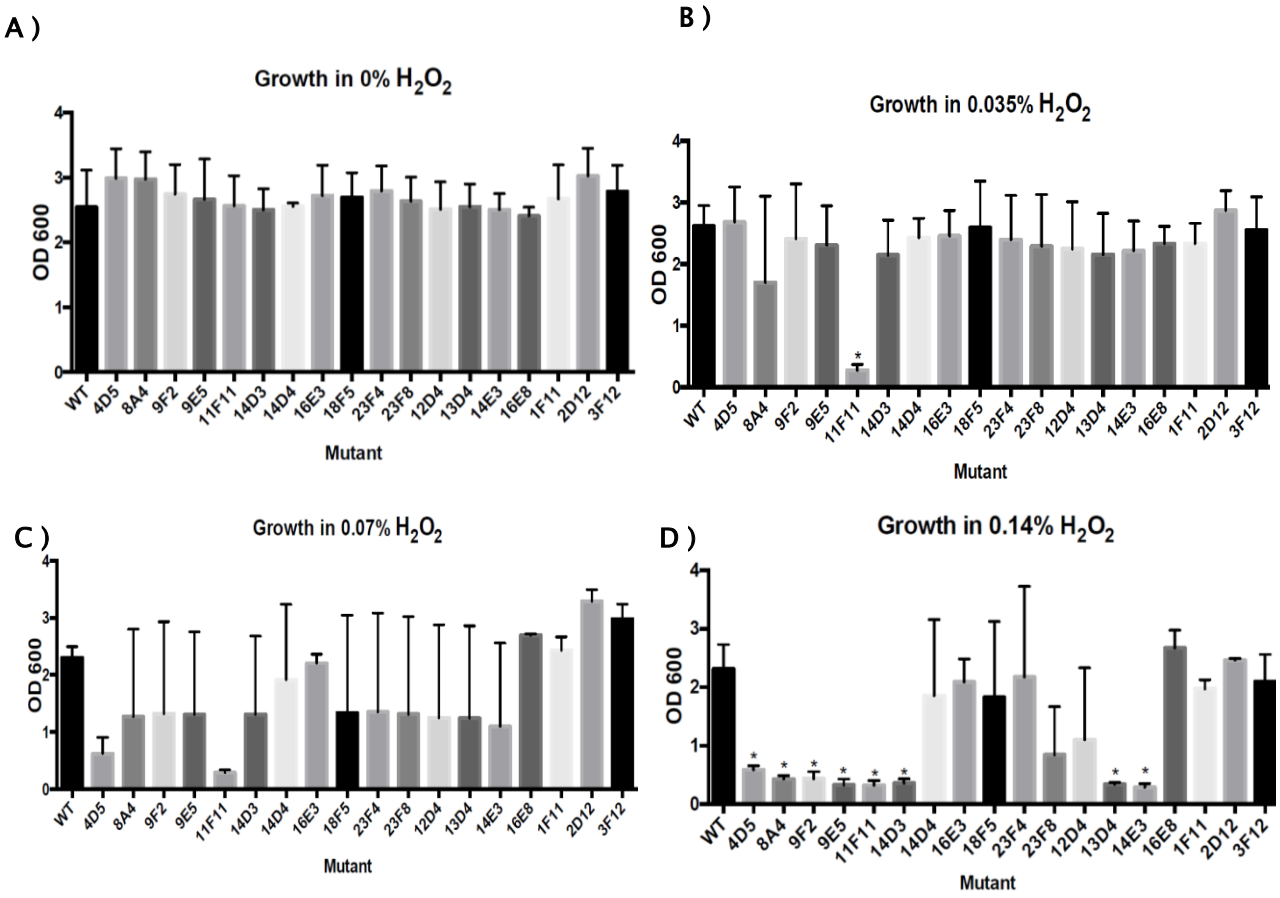

Figure 1. Secondary Screening Identifies 7 Mutants with Susceptibility to H_2O_2. Growth of selected mutants in A) 0%, B) 0.035%, C) 0.07%, D) 0.14% concentration of H_2O_2 in BHI media. The plates were screened three times with the mean OD shown above with error bars indicating standard deviation. In all panels, statistical significance between WT and the mutant is designated with *$p<0.05$ one-way ANOVA followed by Dunnett's multiple comparison post-hoc test.

Use of *Galleria mellonella* infection model to confirm attenuated of mutants of interest.

From the primary secondary screening, mutants 11F11, 13D4, 14D3, and 14E3 were assigned to me for follow-up and testing in the *in vivo* model using *G. mellonella*. The use of this model creates a way to test the virulence of each mutant in a whole-animal model. Death

of the worm is the endpoint, which is clear as the infected worms turn black as seen in Figure 2 Panel B. Worms weighing between 190 and 220 mg were selected for infection, and the average masses among each injection group can be seen in Figure 2 Panel A. Worm masses were approximately equivalent with no statistical difference in weight. This ensures results reflect the difference in the bacterial mutant injected and not differences in the ratio of injected bacteria to worm size. The tested mutants were grown to log phase (optical density of 0.4), washed in PBS, and diluted 1:2. A 10-µL aliquot of each 1:2 dilution of the transposon mutant, wild-type, or saline (PBS) was injected into the corresponding test worms. The starting bacterial concentration for all injections (cfu/mL) was also calculated and found to be roughly equivalent for all strains. The survival of the worms was documented every 24 hours for 3 days. Percent survival of each group of worms can be seen in Figure 2 Panel C. The saline worms were used as a negative control to demonstrate that the injection process was not lethal. As expected, this group demonstrated extremely high survival at about 94%, confirming that the injection process was not overtly traumatic. Larvae injected with wild-type demonstrated relatively low survival around 35%. Mutant 4D5 was used as a positive control for attenuated virulence and demonstrated survival around 65%, similar to a previous study's results [13]. Mutants 13D4 and 14E3 mirror this higher survival percentage at about 70% survival and have statistically significant higher survival than wild-type. Mutants 11F11 and 14D3 demonstrate survival comparable to wild-type at around 30% survival. These results demonstrate that mutants 13D4 and 14E3 not only have attenuation *in vitro* but also overall attenuated virulence in a host.

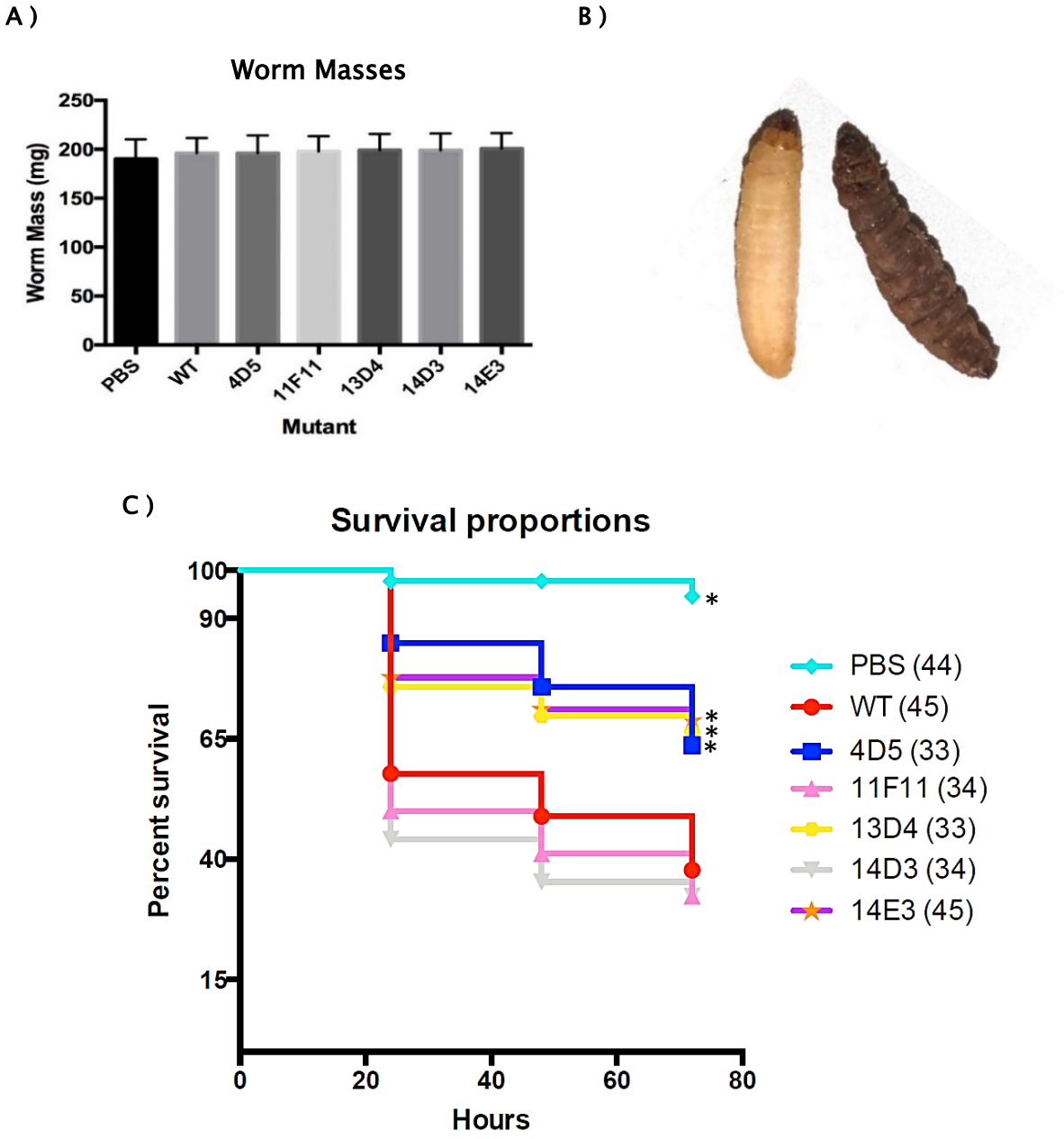

Figure 2. Mutants 13D4 and 14E3 Demonstrate Attenuated Virulence in *G. mellonella*. **A)** Worm masses for each injection group were recorded, and the average mass is shown with error bars indicating standard deviation. **B)** Comparison of a living infected worm (left) and a dead infected worm (right). **C)** Percent survival of worms injected with saline (PBS), the mutants, and wild-type *B. anthracis* at hours 24, 48, and 72 hours. Total number of worms in each injection group are indicated in parenthesis. Significantly different survival compared to WT is indicated *$p<0.05$ by log-rank statistical analysis.

Determination of the transposon location of the mutants of interest.

The next step of this study was to determine the location of the transposon insertion into the genome in order to determine which gene, or genes, was interrupted, leading to the observed phenotype. This was done using the Y-linker method adapted from Kwon and Ricke [25]. This method uses a restriction enzyme, NlaIII, in order to create many cuts randomly within the DNA. The Y-linker is then added to the digested DNA. The Y-linker is comprised of two partially complementary primers that are annealed together (forming the shape of a Y) and have NlaIII compatible sticky ends. The Y-linker is then ligated to the ends of the NlaIII digested genomic DNA. A PCR is performed with the Y-linker specific primer and the transposon specific primer, Himar 1-2 Long, to amplify the section of bacterial DNA that contains both the transposon and a fragment of the *B. anthracis* genomic DNA. Figure 3 shows the results of our PCR reaction. Mutant 13D4 produced a strong band of approximately 500 base pairs; however, the other mutants produced only very small bands that are likely non-specific amplifications caused by primer-dimers. In attempt to get a successful PCR amplification with the other mutants, adaptions in the protocol were made, such as adjustments in amount of DNA template added or changing the primer annealing temperatures using a gradient PCR. However, we were unable to produce a successful PCR of the other mutants. The PCR product of mutant 13D4 was then purified, sequenced, and BLASTed to determine the location of transposon insertion as seen in Figure 4.

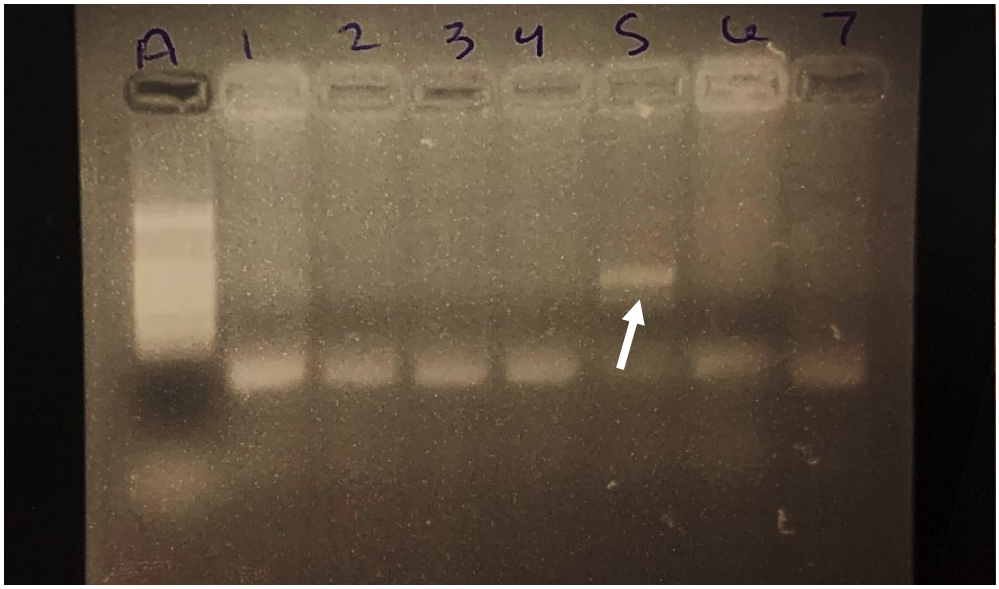

Figure 3. Gel electrophoresis of the successful PCR product of mutant 13D4. The PCR used to amplify the chromosomal location of the transposon in the mutants. Mutant 13D4 is indicated by the arrow.

```
  1 CGGATACCGACCAGGGCTATCAGCCACCTGTTATTATATGGA
 43 AAAGGATTAACAAACGCTGGATGGGTGTTTGGTTCTCACTTC
 85 TTATTACAGTTGCGTACGGATTAATTTTCAACTCTGTACAAG
127 CAAACACAGTAACAATAGCATTTGAAAATGCTTTTGGACTAG
169 AGCGAACGATTGTAGGAGCTCTATTAGCTTTATTAGTTGCAG
211 TTATTATTTTCGGTGGTATTAAGAGCATTTCACGTATTACAG
253 AAATGATTGTTCCGCCAATGGCAATCATTTATATTGGTGTGG
295 CTATTTTTGTCGTGATTAACAACTTCACTATGCTACCAAGCA
337 TTTTTACAGAAATATTTAACAGCGCATTTGGTTTAGACCAAG
379 CCGTCGCTGGTGGTATTGGAGCAGCAATCAAGTTCGGAATTC
421 AGCGCGGTTTATTCGCGACAGAAGCAGGTATGGGTAGC
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Figure 4. Sequencing of 13D4 mutant shows location of transposon insertion into *B. anthracis* genome. The PCR product was sequenced, and the genome was BLASTed to be compared to wild-type *B. anthracis*. The blue highlighted bases represent the known transposon sequence. The following bases align with *B. anthracis* genome. The highlighted green bases indicate the region recognized by our complementary reverse primer. There are 407 bp between the transposon sequence and the sequence that will be used as the mutant specific primer.

This sequenced genome was used to create a reverse primer complementary to a section of the genome 407 base-pairs downstream from the predicted transposon insertion site (blue highlighted region, Fig. 4). This complementary region is seen in Figure 4 as the green highlighted bases. We amplified the genomic DNA from the transposon mutant using the transposon specific primer and the mutant specific reverse primer. As can be seen in Figure 5, the band produced from this PCR matches the 408 base pair band from the ladder and therefore is the predicted size that should be produced using our two primers. This confirms the location of the transposon in 13D4. In Figure 5, wild-type was also included in the PCR as a negative control. Wild-type does not include the transposon sequence, and therefore, no amplification of DNA should occur. If amplification occurred and a band was produced, this would indicate that our primers are not specific to the sequence in mutant 13D4, and therefore are not amplifying the transposon insertion site. However, no band was seen, confirming that the amplification is specific to the transposon insertion site.

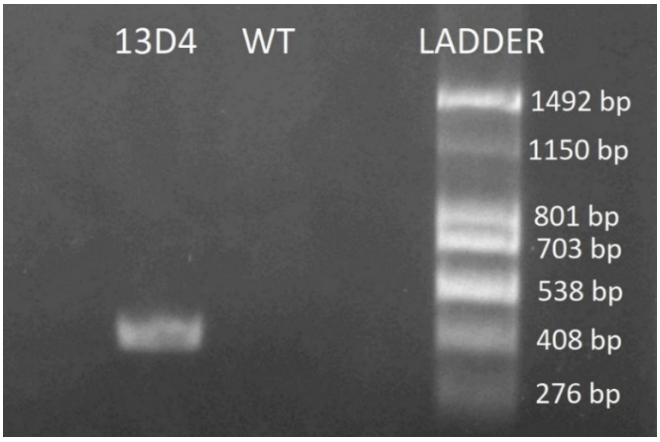

Figure 5. PCR confirmation of 13D4. PCR was performed with transposon specific primer, Himar 1-2 Long, and a site downstream of the transposon insertion, 13D4 primer. A band is seen at the expected size of 407 bp.

The location of the transposon insertion can be seen in Figure 6, which is a schematic of the operon and genes found near the insertion site. The transposon is inserted into the gene AAT57204.1, which is a sodium/alanine symporter. This gene is followed by the genes AAT57205.1 and AAT57206.1, which correlate to a general stress protein 20U and a hypothetical protein, respectively. Due to the proximity of these genes in the genome, these 3 genes are most likely regulated together as an operon and may all be affected by the insertion of the transposon.

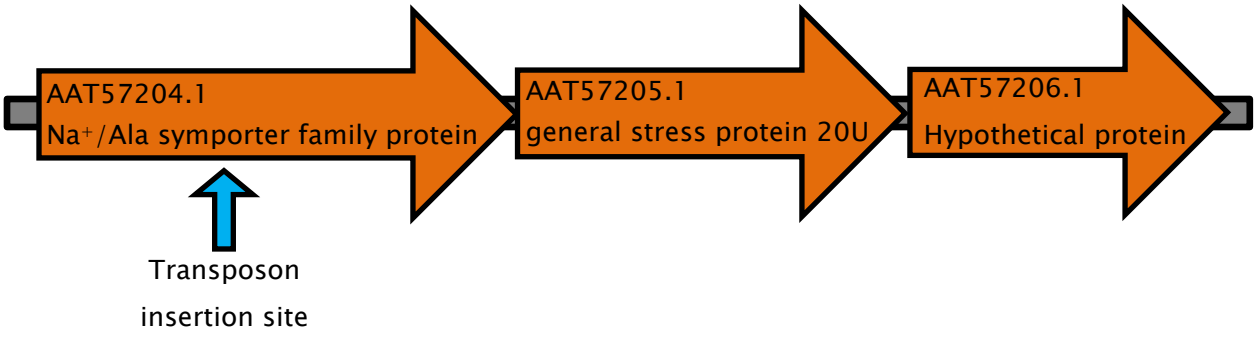

Figure 6. Genome structure of affected genes of mutant 13D4. This figure demonstrates the approximate location of transposon in the middle of gene AAT57204.1 and operon.

DISCUSSION

Overall, the primary screen method and protocol development worked efficiently and effectively as we had hoped. It allowed for a quick and high rate of screening, permitting us to screen almost 2,000 transposon mutants. From this primary screen, we were able to pull out 40 mutants of interest, giving hit percentage of around 2%, which is comparable to the hit-rate

from previous productive screens conducted with this library in our lab [17, 18]. The secondary H_2O_2 screen confirmed 7 out of 17 mutants as having a consistent *in vitro* phenotype. This is lower than we initially expected, since all 17 of these mutants were attenuated in our primary screen. However, given the volume of mutants we were working with in our primary screen, it is possible conditions were not as consistent as they were in the secondary screen. For example, our secondary screen was not performed at the same concentration as the primary screen. In the primary screen, serial 1:2 dilutions of the hydrogen peroxide media were used to preliminarily test the protocol on the mutants to determine the concentration that would be used. The choice of which concentration was most appropriate to pull out mutants of interest was determined by our controls, wild-type and 4D5, and was therefore 0.035% H_2O_2. In the secondary screen, the serial 1:2 dilutions of the hydrogen peroxide media were repeated in each trial giving three different concentrations that the mutants were all tested in. This data showed that 0.14% hydrogen peroxide media gave the most reliable and consistent results between mutants of interest and wild-type like mutants. Theoretically, these values should not differ, as the conditions and mutants did not change. However, these screens were done months apart, and the H_2O_2 stock may be degraded slightly, or minor changes in the media could have affected results. Growing *B. anthracis* Sterne in the small volume (200 µL) found in 96-well plates is also not ideal, as small differences in conditions are more likely to lead to large differences in growth. Further optimization of the secondary screen is likely still needed, especially given the large error bars found in several of the mutants, including 12D4, 14D4,

18F5, 23F4 and 23F8. Nonetheless, we successfully pulled out 7 mutants consistently attenuated for growth in H_2O_2 in our in vitro assays.

As expected, not all of our *in vitro* mutants produced an *in vivo* phenotype of attenuated virulence. In our *in vitro* method, we screened solely for hydrogen peroxide susceptibility, but this is not a full depiction of the virulence of *B. anthracis* as its interactions with a true host are much more complicated. Though some of the mutants may be more susceptible to H_2O_2, they may still be able to fully establish an infection. Therefore, using an *in vivo* screen provides a more direct look at the attenuated virulence of *B. anthracis* and allows us to concentrate on mutants that are more likely to have an interruption that is relevant to an infection of a host. Overall, this dual-screening method allows us to focus on the two aspects of a *Bacillus anthracis* infection that we were interested in- resistance to hydrogen peroxide used by the immune system and the bacteria's strong ability to establish an infection in hosts. Mutants 13D4 and 14E3 were pulled out as having attenuation in both screens, and therefore are mutants of interest that should be investigated to determine the transposon insertion site. We were able to determine the insertion site in mutant 13D4, but unable to replicate this success in the mutant 14E3. However, this mutant still demonstrated clear attenuated growth and virulence, and therefore will be followed-up by a later student in the lab in order to determine the transposon insertion site and characterize the mutant.

We found mutant 13D4 to contain the transposon interruption in a gene for a sodium/alanine symporter family protein. In addition, two other genes follow closely to the interrupted gene, indicating that these three gene are likely in a single operon and may also be

interrupted by the transposon's insertion. These two genes following the interrupted gene correlate to a general stress protein 20U and a hypothetical protein, respectively. Sodium/alanine symporters are common among many bacteria, including a number of pathogenic species [26]. The proton motive force is integral to the survival of all living species and is created and utilized through many primary and secondary transporters, including sodium/alanine symporters. While *B. anthracis* is auxotrophic for alanine [27], alanine may have a deeper importance to the overall sodium cycle and maintenance of the proton motive force of the species. In addition, alanine and its interconversion between the D and L stereoisomers is also integral to sporulation and germination of *B. anthracis* [28]. Neither of these functions directly explain what relationship, if any, there is between virulence and a non-functional sodium/alanine symporter family protein but may offer a possible route for investigation.

Interestingly, general stress protein 20U, also known as Dps2, has been linked to bacterial regulation of iron and hydrogen peroxide [29]. Many pathogenic bacteria require the acquisition of iron from their host environment in order to grow and establish an infection. Dps is a family of proteins conserved among many pathogenic bacteria that utilize H_2O_2 to facilitate the oxidation of iron from Fe(II) to Fe(III) for store and use [29]. While other ferritins exist to acquire iron, they utilize different molecules as the oxidizers. Dps proteins offer an advantage in that their acquisition and oxidation of iron also depletes the extracellular H_2O_2 through this process [29]. By consuming the H_2O_2, this allows a higher hydrogen peroxide tolerance for the bacteria. As the immune system utilizes hydrogen peroxide as one of the first lines of defense against pathogens, this may also confer higher virulence to a bacterium with functional Dps

proteins as it can tolerate the elevated hydrogen peroxide levels through the use of the Dps protein. However, the disruption of such a protein would not only hinder the bacterium's ability to acquire iron but also its defense against the immune system's utilization of hydrogen peroxide. This mechanism could potentially explain the susceptibility of mutant 13D4 to hydrogen peroxide, as the general stress protein 20U gene may also be affected and rendered nonfunctional.

Bacillus anthracis possesses two varying copies of the Dps protein [29]. These proteins are not identical with an alignment of about 61%. While both function as Dps proteins, Dps2 has been shown to be one of the highest iron-containing proteins in the cytoplasm, possibly indicating higher functionality [30]. However, it has also previously been demonstrated that the deletion of the Dps2 gene does not affect the survivability of *B. anthracis* in either iron-rich or depleted environments [30]. This most likely indicates that the main function of this protein is the management of oxidative stress for the bacterium rather than simply iron acquisition [30]. While the correlation of the Dps2 gene and peroxide resistance is not novel, our data expands on this previous knowledge of the importance of the gene Dps2 and indicates that the Dps2 protein also shows a direct relation to the virulence of *B. anthracis*. Dps2 is not only critical to the resistance of peroxide species but also to the establishment of an infection in the host. In the future, this mutant should be confirmed through the creation of a targeted Dps2-deficient mutant. The mutant would then be expected to present with the same attenuation found in mutant 13D4 but could be restored to wild-type virulence and phenotype through the introduction of a plasmid containing the Dps2 gene. If this was confirmed, then we will have

established that the Dps2 gene is an integral part of the *Bacillus anthracis* infection. In addition, because of the success of the isolation of mutant 13D4, the other mutants of interest should also be characterized in the hopes of determining further genes essential to *B. anthracis*'s virulence.

In conclusion, this research project has successfully established screening conditions that can identify highly relevant, potential virulence genes. Two mutants, 13D4 and 14E3, as well as all four of the other mutants that were handed off to lab member Taylor Kelly, have shown strong promise in our *in vivo* assays and should be further characterized. This success confirms the attainment of our study's goal to pull out interesting *B. anthracis* mutants for the purpose of determining genes essential to its establishment of infection. The identification of a previously confirmed peroxide tolerance gene, Dps2, that is likely disrupted in mutant 13D4, further validates our study's model and indicates that our other mutants of interest may also contain interruptions in genes, that once characterized, may prove to be novel and vital to *B. anthracis*'s ability to establish an infection. By continuing with the study to characterize the other mutants, we may be able to further understand the mechanism by which *B. anthracis* is able to efficiently establish infection in hosts. This is important not only to understanding the basic pathogenesis of B. anthracis, but also may represent potential drug targets for combatting *B. anthracis* or other bacterial infections.

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