DISCOVERY OF NOVEL VIRULENCE MUTANTS BY ASSESSING HYDROGEN

PEROXIDE SENSITIVITY IN BACILLUS ANTHRACIS

by

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ABSTRACT

Bacillus anthracis, the causative agent of anthrax and a Disease Category A priority pathogen, utilizes an array of chromosome-encoded virulence factors to subvert the host immune response. After screening a pool of randomly generated *B. anthracis* Sterne mutants, we identified a mutation in the gene *ywlE* that rendered the bacteria more susceptible to hydrogen peroxide than the wild-type. In *Bacillus subtilis*, YwlE plays a role in modulating the stress response (Musumeci *et al.,* 2005). However, experiments confirming this phenotype with an insertional mutant revealed that YwlE does not independently regulate the oxidative stress response in *B. anthracis.* Concurrently, we investigated the larva of the greater wax moth *Galleria mellonella* as a model for *B. anthracis* infection that would be able to provide an *in vivo* demonstration of decreased virulence with the knock-out of YwlE. This organism has shown promising results as an infection model for other human bacterial pathogens, such as *Staphylococcus aureus* (Purves *et al.*, 2010), leading us to test its usefulness in *B. anthracis* studies. We show that while *B. anthracis* Sterne does infect *G. mellonella*, infections with *B. anthracis* Sterne strains of varying degrees of virulence in mammalian models does not correlate with *G. mellonella* survival rates, confirming that this is not an effective model organism for studying *B. anthracis* virulence.

ACKNOWLEDGMENTS

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INTRODUCTION

Bacillus anthracis is a gram-positive, spore-forming bacteria and the causative agent of anthrax. Infection is most common in domesticated herbivores, but transmission to humans is possible if contact is made with infected animals or contaminated animal products. Cutaneous anthrax is the most common form of the disease and is curable when treated with antibiotics. Inhalational anthrax, resulting from the inhalation of *B. anthracis* endospores and subsequent septicemia, has a mortality rate of greater than 80% (1). The ability of the endospores to rapidly germinate in the macrophage, escape the phagolysosome, and spread to the lymph nodes, circulatory system, and central nervous system are what make *B. anthracis* a Biodefense Category A Priority Pathogen. A push for further investigation of *B. anthracis* pathogenesis followed the 2001 anthrax attacks that infected 22 Americans, killing five (2).

B. anthracis virulence is largely attributed to plasmids pX01 and pX02. The precursors of edema toxin and lethal toxin are encoded on pX01 and together make up the anthrax exotoxins. Edema toxin indirectly contributes to virulence by inhibiting function of neutrophils and macrophages and interfering with the inflammatory response (3). The cytolytic properties of lethal toxin target macrophages, increasing levels of IL-1β and inducing septic shock in the host (4). Multiple proteins necessary for the synthesis and regulation of the antiphagocytic capsule are encoded on pX02 (4). Despite the differences in the roles of pathogenesis each plasmid plays, a deficiency in one or both of these virulence plasmids renders the strain nonvirulent in most hosts (4).

While these plasmids are the best-characterized contributors to host disease and death, whole-genome sequencing of fully-virulent *B. anthracis* Ames revealed that the

bacteria possesses multiple chromosome-encoded proteins that are homologous to known virulence factors in the highly related species *Bacillus cereus* and *Bacillus thuringiensis* (5). In order to identify novel chromosome-encoded virulence genes in *B. anthracis*, my mentor Dr. Shauna McGillivray created a library of mutant *B. anthracis* strains via transposon-based mutagenesis (6). This method exposes the bacterial chromosome to short pieces of DNA known as transposons. The transposons possess the ability to insert into the genome in random places, inducing a random mutation each time. Thousands of progeny bacteria are created as a result, each possessing a different mutation caused by the random insertion of a transposon.

Once a library has been created, candidates with mutations in potential virulence genes must be identified through the use of screens that compare the phenotype of the particular mutant strain against wild-type *B. anthracis*. *In vitro* screens mimicking host conditions outside of a live organism can provide the means for quantifying common indicators of virulence and are advantageous because of their ease of use and high throughput nature. For example, hemolytic activity, or lysis of red blood cells, is often an indicator of virulence: not only does it enhance bacterial survival by enabling iron acquisition, but it also indicates general cytolytic abilities. Because anthrax is known to indirectly induce hemolysis (7), measuring the relative *in vitro* hemolytic activity of a particular transposon mutant can indicate genes necessary for this mechanism of disease. Indeed, loss of hemolytic activity *in vitro* was linked to a mutation in the ClpXP ATPase gene of *B. anthracis*, a gene later shown to be essential for virulence in mouse models of infection (6). Additional high throughput *in vitro* screens include assessing proteolytic activity, a *B. anthracis* virulence phenotype caused by numerous caseinolytic and

collagenolytic bacterial proteases (8). *In vivo* models can be useful for initial screening of potential virulence genes, but are limited to invertebrate models such as the nematode *Caenorhabditis elegans* (9). Use of *C. elegans* has identified a collection of possible *B. anthracis* virulence factors. In particular, a cluster of tellurite resistance genes encoded on the *B. anthracis* chromosome was identified as important for resistance to host reactive oxygen species and for full virulence in a mouse model of infection (10).

In this study, we collected a total of 44 transposon mutants that had previously displayed attenuation in one of three screens: proteolysis, hemolysis, and *C. elegans* lethality. To further narrow down this pool of 44 mutants, we applied a hydrogen peroxide (H_2O_2) survival assay. H_2O_2 falls within a class of chemicals known as reactive oxygen species (ROS) which are released by cells of the innate immune system. Without detoxification mechanisms, ROS cause damage to bacterial genetic material, proteins, and lipids (11). Bacteria have evolved ways to subvert the damaging effects of these chemicals by possessing various catabolic pathways for the breakdown of ROS. The purpose of our H_2O_2 *in vitro* screen of this mutant library was to identify genes that play more than one role in *B. anthracis* virulence by conferring resistance to H_2O_2.

In addition to investigating ROS susceptibility, we wanted to test the virulence of the mutant library *in vivo*. *In vitro* studies provide a critical preliminary screen necessary for identifying the phenotype in question, but they fail to replicate the same pressures a pathogen would face in a mammalian host. This gap can be remedied through the use of *in vivo* models—vertebrate or invertebrate organisms—that possess innate immune systems which force the pathogen to act similarly to the way it might in a mammalian host. The common laboratory mouse *Mus musculus* and the nematode

Caenorhabditis elegans, mentioned above, are infection models that have been used in the past for studying *B. anthracis* virulence. However, *in vivo* models come with their own set of disadvantages. Infection of mice frequently raises ethical and financial concerns that limit their use. *C. elegans* has successfully been used as a model for several pathogens (9) including *B. anthracis* (12), but it requires various manipulations in order for infection to occur which confound its utility as an animal model. Therefore, there is a need for a practical, inexpensive model organism for *B. anthracis* infection.

The larva of the greater waxworm *Galleria mellonella* has shown promising results as an infection model for human bacterial pathogens, such as *Staphylococcus aureus* (13). The larva possesses a well-developed innate immune system, can be infected easily via injection of the pathogen beneath the larva's cuticle (skin), and can incubate at the human body temperature of 37 °C (14), all of which make it a better invertebrate model of infection than the frequently used *C. elegans*. In addition, *G. mellonella* larvae are widely abundant and inexpensive, making them ideal over *M. musculus*. As *G. mellonella* has never before been used to study *B. anthracis*, we wanted to determine its effectiveness as a model organism in the context of bacterial virulence.

My project consists of two major aims: 1) to identify novel genes involved in resistance to oxidative stress; and 2) to assess the effectiveness of *Galleria mellonella* larvae as an infection model with which we can potentially test the virulence of the strains identified in Aim 1 *in vivo*.

EXPERIMENTAL PROCEDURES

Bacterial strains and culture conditions

     Bacillus anthracis Sterne strain (pX01$^+$ pX02$^-$) was grown in Brain-Heart-

Infusion (BHI) media broth or on agar plates at 37 °C in aerobic conditions.

Generation of YwlE-deficient B. anthracis *insertional mutant ΔYwlE.*

     Genomic DNA was isolated from *B. anthracis* Sterne using colony PCR (1 colony

was added to 10 μL of water and microwaved for 1 min) and a 400-base pair region of

ywlE was amplified by polymerase chain reaction with *ywlE*-specific forward primer (5'-

TACAGTGAATTCGGGCACTTGGAAATAGGGTTTCACAT-3') containing a site for

digestion by restriction enzyme EcoRI and *ywlE*-specific reverse primer (5'-ACTACTG

GATCCTTCTGTCATTGTTACTATAATATCTGCCCAATC-3') containing a site for

digestion by restriction enzyme BamHI. Temperature-sensitive, erythromycin-resistant

plasmid pHY304 and the 400-base pair PCR product were individually digested with

EcoRI and BamHI and subsequently ligated together and transformed into

electrocompetent *E. coli* strain M1061 (Lucigen). Colonies were grown on BHI + 500

μg/mL erythromycin at 30 °C and screened using pHY304-specific forward (5'-ACGAC

TCACTATAGGGCGAATTGG-3') and reverse (5'-CCGCTCTAGAACTAGTGGATC

CCC-3') primers. The ligated plasmid was then isolated, purified, and transformed into

electrocompetent methylation-deficient *E. coli* strain GM2163 because only non-

methylated DNA may be transformed into *B. anthracis*. Transformation was selected for

and confirmed as described above, and the plasmid was isolated and purified. The

plasmid was transformed into electrocompetent *B. anthracis* and grown on BHI + 5

µg/mL erythromycin at 30 °C. Temperature shifting to 37 °C in BHI + 5 µg/mL erythromycin forced integration of plasmid into chromosome. Successful integration was confirmed with pHY304 forward primer and *ywlE*-confirm reverse primer (5'-GATCCG CCAAATGGGTCTG-3').

Extraction of genomic DNA

12 mL of *B. anthracis* transposon mutant 45F1 grown overnight to stationary phase was pelleted and resuspended in 755 µL of protoplast buffer (20% sucrose, 20 mM Tris-HCl [pH 7.0], 10 mM $MgCl_2$, 0.05% Triton X 100, and double-distilled water to 10 mL). Lysozyme (100 mg/mL) was added to a final concentration of 20 mg/mL and the reaction incubated for one hour at 60 °C. 25 µL of 20% SDS + 10 µL RNase A (10 mg/mL) were added and the reaction incubated for one hour at 37 °C. 10 µL of 20 mg/mL proteinase K was added and the reaction incubated at 50 °C for one hour. An equal volume of phenol/chloroform was added (1 mL) and the mixture was spun for five minutes at 10000 rpm. The upper aqueous layer was removed and set aside. Phenol/chloroform was added again, the mixture was spun, and aqueous layer was once again removed. 3M sodium acetate and isopropanol were added (0.10 and 0.60 volumes, respectively) to aqueous layer containing DNA and reaction was gently mixed until the DNA precipitated. DNA was pelleted by spinning for five minutes at 10000 rpm, washed with 500 µL 70% ethanol, and spun again. Ethanol was removed and pellet dried at room temperature for approximately ten minutes. Pellet was resuspended in 100 µL of water.

Identification of transposon insertion site

The protocol described by Kwon & Ricke, 2000 was used to identify the sequence flanking the transposon insertion (15). Genomic DNA was extracted from transposon mutant 45F1 using phenol/chloroform as described above and digested with restriction enzyme NlaIII for three hours. 18 µL of 100 µM "linker 2" (5'-TGTCCCCGTACATCGT TAGAACTACTCGTACCATCCACAT-3') was phosphorylated with 2 µL of PNK, 4 µL of 10X T4 ligase buffer (NEB) and 16 µL water at 37 °C for one hour, and then heat denatured at 65 °C for 20 minutes. A Y-shaped primer containing an NlaIII digestion site overhang was generated by annealing 18 µL of 100 µM "linker 1" (5'-TTTCTGCTCGA ATTCAAGCTTCTAACGATGTACGGGGACCATG-3') to heat denatured "linker 2" by heating to 95 °C for five minutes and then allowing the reaction to return slowly to room temperature. Approximately 200 ng of digested DNA was ligated overnight to 5 µL of Y-linker with 1 µL of T4 DNA ligase (NEB) to a final concentration of 20 µL. Reaction was diluted to a final volume of 200 µL and the T4 DNA ligase was denatured by heating to 65 °C for ten minutes to denature T4 DNA ligase. 2 µL were used for template and amplified with a Y-linker specific primer (5'-CTGCTCGAATTCAAGCTTCT-3') and a transposon-specific primer Himar 1-2 long (5'-GGGAATCATTTGAAGGTT-3') by PCR. The PCR product was gel purified and sequenced with Himar 1-4 primer (5'-TATG CATTTAATACTAGCGAC-3'). The sequenced portion of the genome was identified using a BLAST search.

Hydrogen peroxide survival assays

For the initial hydrogen peroxide screen of 44 *B. anthracis* transposon mutants, bacteria from frozen stocks were grown in static conditions overnight in BHI at 37 °C in a 96-well plate. After 24 hours of growth, bacteria were diluted 1:100 in 200 μL BHI + H_2O_2 (0%, 0.01%, 0.02%, or 0.04%) and incubated overnight in the same conditions as above. After approximately 24 hours, optical density was measured (600 nm). The data from 3 independent experiments were combined and mutant growth was normalized to WT by calculating the percentage of the optical growth of the mutant at 0.01% H_2O_2 compared to the optical growth of WT *B. anthracis* at the same concentration. For larger scale assays used to compare the response of WT *B. anthracis* transposon mutant B2 and insertional mutant ΔYwlE, bacteria were grown to log phase in shaking conditions and diluted 1:10 in BHI + H_2O_2 (0%, 0.005%, 0.0075%, or 0.015%). Optical density (600 nm) was measured after approximately 24 hours. Data represents three independent experiments. Analyses performed included one-way ANOVA followed by Dunnet's post hoc test.

Galleria mellonella *haemocoelic injections*

The following procedure was modified from Ramarao *et al.*, 2012 (14). *Galleria mellonella* larvae in the last larval stage (during which they no longer feed and begin to produce silk) were ordered from Waxwormstore.com and stored at 4 °C until use. For the *B. anthracis* dilution survival assay, *B. anthracis* Sterne was grown to log phase (optical density [600nm] = 0.4), spun down and resuspended in an equal volume of PBS. Worms were placed at room temperature (about 23 °C) for 24 hours prior to infection. Each

treatment group contained 9-11 waxworms with an average mass of 232 mg. An injection of 20 μL containing 1.5 x 10^5 cfu was administered to the undiluted infection group. The bacteria was diluted ten-fold in PBS and 20 μL containing 1.4 x 10^3 cfu were injected into the 1:10 dilution infection group. Two more ten-fold dilutions were made and 20 μL containing less than 1 cfu (i.e. bacteria undetectable by dilution plating) were injected in to the 1:100 dilution group and 1:1000 dilution group, respectively. Injections were administered by hand with a 1-mL hypodermic syringe through the posterior cuticle (haemocoelic injection). Worms were incubated at 37 °C following infection and survival was recorded at approximately 24 and 48 hours post infection. For the comparison of survival between *B. anthracis* (wild-type and ΔpX01) and *B. subtilis*, each treatment group contained 10 larvae with an average mass of 145 mg. Larvae were kept at 4 °C until injection was administered to reduce movement. Bacteria strains were grown to log phase. *B. anthracis* WT and ΔpX01 were diluted 1:5 in PBS and *B. subtilis* was diluted 1:50 in PBS. Bacteria from each treatment group were enumerated by serial dilution plating. Worms received a total of 10 μL haemocoelic injection administered by an automated pump (New Era Pump Systems NE-500) set to administer 2.5 μL per injection. The WT *B. anthracis* inoculum contained 5.2 x 10^4 cfu, the ΔpX01 *B. anthracis* inoculum contained 2.4 x 10^4 cfu, and the WT *B. subtilis* inoculum contained 3.6 x 10^4 cfu. Worms were incubated at 37 °C for 48 hours beginning at time of infection. Survival was recorded at 24 and 48 hours post infection. Data was plotted using a Kaplan-Meier Survival plot.

<u>RESULTS</u>

Transposon mutants displaying decreased virulence also show increased susceptibility to hydrogen peroxide.

Previous work identified 44 *B. anthracis* Sterne transposon mutants that displayed decreased virulence in *in vitro* or *in vivo* virulence screens (6, 9, and data not shown). Of the 44 mutants, 13 displayed increased *C. elegans* survival, 19 displayed low proteolytic activity, and 12 displayed altered hemolytic activity (9 and unpublished data). In order to assess further evidence of decreased virulence, we investigated the ability of the transposon mutants to grow in the presence of hydrogen peroxide. H_2O_2 is one of several chemicals created by the release of superoxide anions within the macrophage to initiate oxidative stress and subsequent burst in phagocytosed pathogens (10). *B. anthracis*, like many bacterial species, has evolved ways to subvert the consequences of oxidative stress by encoding enzymes such as superoxidase and catalase, which break down these harmful chemicals, and by initiating sporulation within the phagosome for protection (17). We applied a H_2O_2 survival assay to determine how the transposon mutants might behave in the presence of host ROS. We first determined the minimum inhibitory concentration for growth of WT *B. anthracis* Sterne to be 0.02% H_2O_2 (data not shown). Mutants were grown overnight to stationary phase and treated with 0.01% H_2O_2 for approximately 24 hours, after which optical density was measured to determine relative growth (Fig 1). Nine mutants fell below the 35% survival relative to WT growth at the same concentration of H_2O_2 (Table 1). Of these 9 mutants, the site of the transposon insertion had already been discovered for four. Mutant B11, displaying 17.8% relative growth, possesses a disruption in a tellurium resistance gene and has previously shown
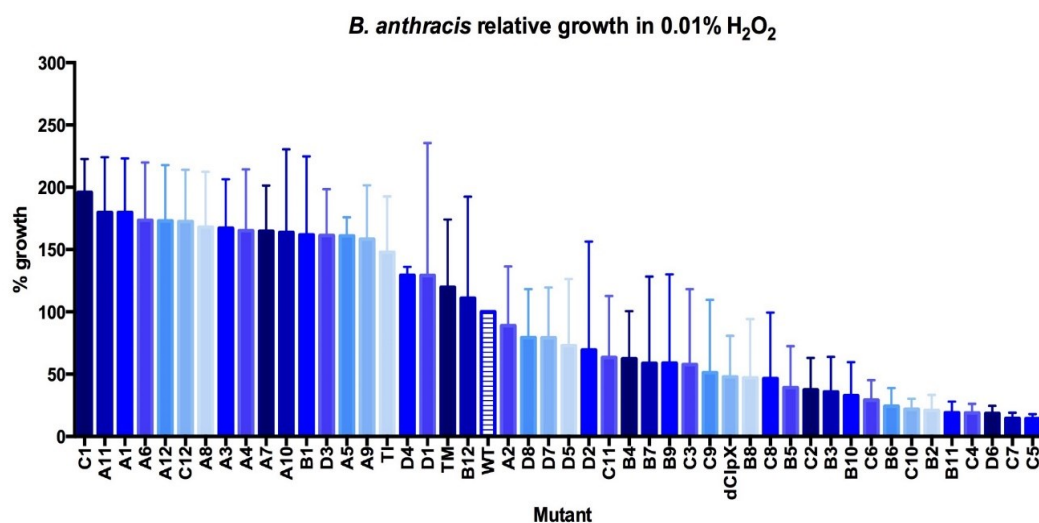
Figure 1 *B. anthracis* Sterne *t*ransposon mutants show varied susceptibility to hydrogen peroxide. WT and transposon mutant *B. anthracis* Sterne grown to stationary phase and treated with 0.01% H_2O_2. Optical density was measured after ~24 hours. Data is the combined results of three independent experiments.

| ID	Relative survival (%)	Gene disrupted
C7	14.0	Unknown
C5	14.1	Unknown
D6	17.6	Sphingomyelin phosphodiesterase C
C4	17.8	Unknown
B11	17.8	Putative tellurium resistance gene
B2	19.1	Protein tyrosine phosphatase YwlE
C10	22.8	Petrobactin biosynthesis protein AsbC
B6	23.7	Unknown
C6	30.8	Unknown

Table 1 Transposon mutants demonstrating significant attenuation and their respective mutations. Mutants that fell below a threshold value of 35% growth with regards to WT *B. anthracis* Sterne when treated with 0.01% H_2O_2 were considered the most attenuated. The location of the transposon insertion was previously identified for about half of the selected mutants. Mutants that underwent further study for reasons described in the text are highlighted.

H_2O_2 susceptibility (9), further validating our results. Thus, we were able to conclude that several transposon mutants with defects in virulence-associated phenotypes also show evidence of a disruption in their ability to regulate oxidative stress.

YwlE does not independently affect the resistance of B. anthracis *Sterne to hydrogen peroxide.*

We chose to confirm the results seen in transposon mutant B2, which possessed a disruption in the gene coding for low molecular weight protein tyrosine phosphatase YwlE. The role of YwlE has not been investigated in *B. anthracis* but previous studies show its role in facilitating the stress response in *B. subtilis* (18, 19, 20). We created an insertional mutant by targeted insertion of an approximately 4 kbp-plasmid into the gene *ywlE* (Fig 2A). This disruption was confirmed via polymerase chain reaction using a forward primer specific for the plasmid and a reverse primer specific for genomic DNA downstream of the insertion site (Fig 2B). To confirm the results seen with the transposon mutants, we repeated the H_2O_2 survival assay using WT *B. anthracis* Sterne, the transposon mutant B2, and the insertional mutant ΔYwlE. Minimum inhibitory concentration of H_2O_2 for WT *B. anthracis* Sterne grown to log phase was determined to be 0.01%, due to the lower density of bacterium in the culture (data not shown). WT, transposon mutant, and insertional mutant were grown to log phase and treated with 0.0075% H_2O_2. After 24 hours, optical density was measured. WT *B. anthracis* Sterne and the transposon mutant displayed results consistent with the original stationary phase analysis of growth in H_2O_2, in that the transposon mutant displayed 11.6% growth;
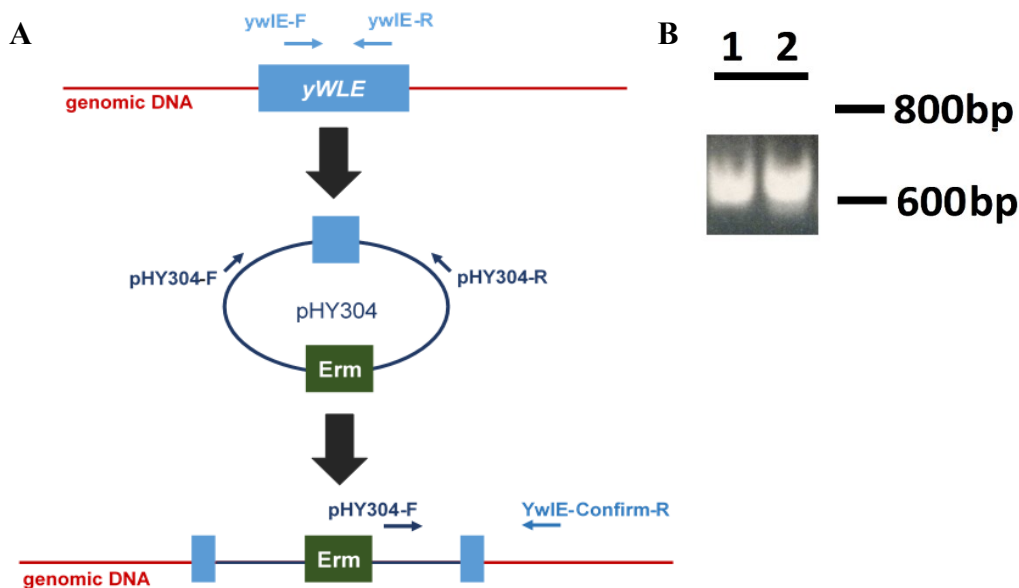
Figure 2 Creation of a YwlE insertional mutant. (A) A 400-base pair region of YwlE was amplified by PCR and inserted into plasmid pHY304. Electrocompetent *B. anthracis* cells were transformed and the plasmid was integrated into the chromosome through homologous recombination. Transformed cells were selected for in 5ug/mL erythromycin + BHI. (B) Successful insertion of the plasmid into the chromosome was screened in two individual colonies by PCR using a pHY304-specific forward primer and a YwlE-specific confirmation reverse primer. Amplified region was detected by gel electrophoresis.
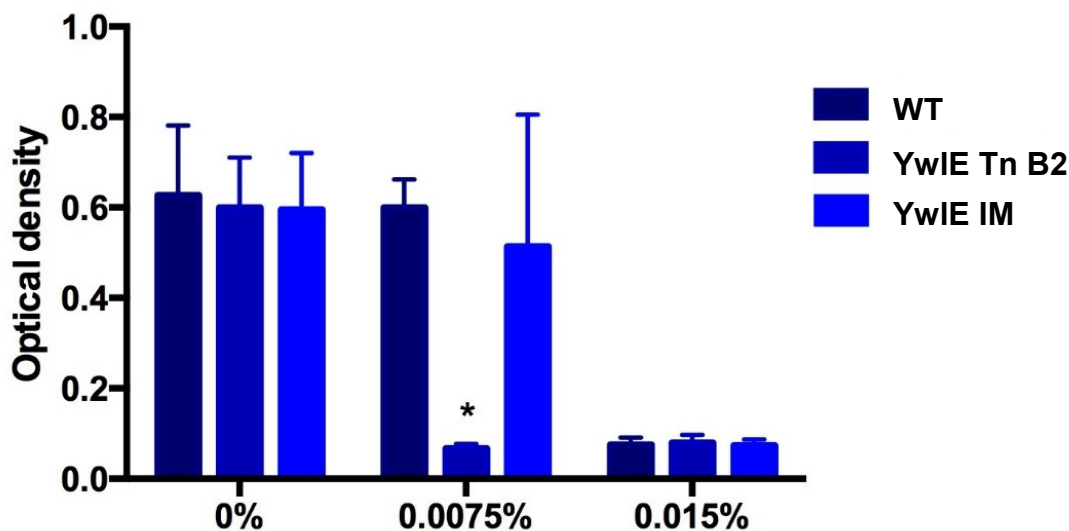
Figure 3 YwlE is not necessary for the protection of *B. anthracis* against hydrogen peroxide. Log-phase WT, transposon mutant B2, and insertional mutant ΔYwlE *B. anthracis* treated with 0.0075% and 0.015% hydrogen peroxide. Optical density (600nm) measured after ~20 hours. Analyses performed included one-way ANOVA followed by Dunnet's post hoc test. Asterisk indicates $P < 0.05$. Data is the combined results of three experiments.

however, the ΔYwlE insertional mutant grew comparable to WT *B. anthracis* Sterne (Fig 3). Further investigation using a YwlE complement vector is currently in progress to determine if the transposon mutant possesses another unknown mutation causing the phenotype in question.


Figure 4 Larvae of the wax moth *G. mellonella*. *G. mellonella* larvae injected with PBS (left) are still alive and can be easily distinguished from dead worms (right) injected with *B. anthracis*.

Galleria mellonella *is not an effective model for the study of* B. anthracis *Sterne pathogenesis.*

Ramarao *et al.*, 2012 reported on the effectiveness of *Galleria mellonella* larvae for studying the pathogenesis of several bacteria including *Proteus vulgaris* and *Listeria monocytogenes*, and the waxworm has been used to study virulence in *Staphylococcus aureus* (14, 13). We investigated the effectiveness of the worm to serve as an invertebrate model organism for *B. anthracis* Sterne infection. We found that injection of *B. anthracis* Sterne resulted in a successful infection of the waxworm in comparison to the PBS control (Fig 4). Next, we determined the dose of *B. anthracis* Sterne which would results in 50% lethality to the worms (LD50). We administered serial 10-fold dilution of *B. anthracis* Sterne to the waxworms and found that worm survival was dose-dependent and the LD_{50} was approximately 1.4 x 10^3 cfu (Fig 5). In order to assess if *G. mellonella* survival can be used as a measure of virulence of a particular strain of *B. anthracis*, we compared the survival rates between a
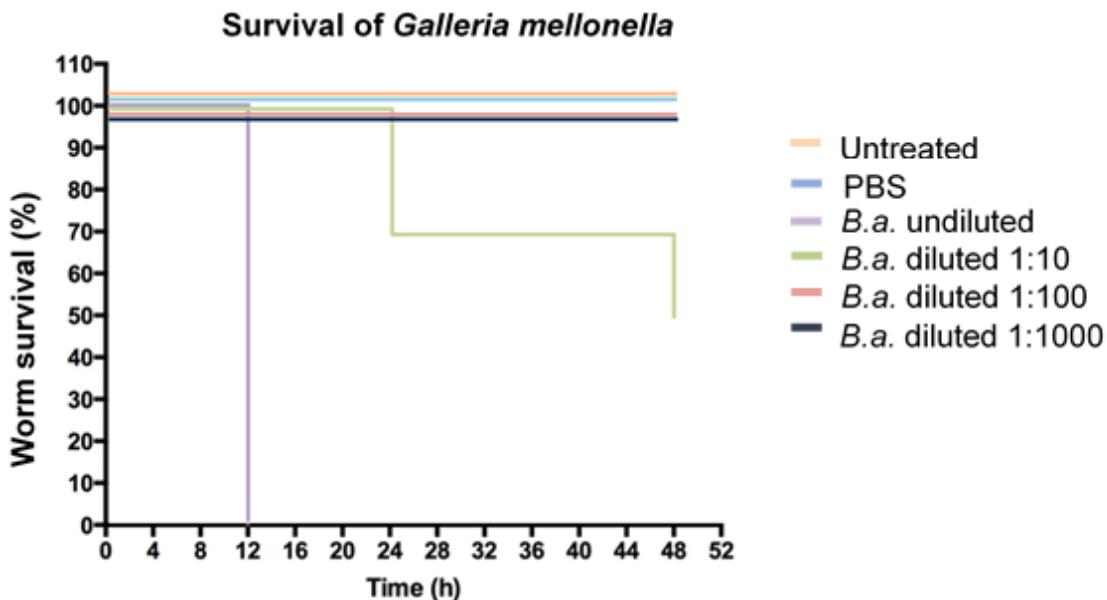
Figure 5 *B. anthracis* Sterne kills *G. mellonella* larvae in a dose-dependent manner. *G. mellonella* larvae received intrahaemocoelic injections of log phase *B. anthracis* Sterne diluted in PBS to the concentrations shown. Injections were administered at 0 hr. Worms incubated at 37°C and percent survival was recorded ~24 and ~48 hours. Each infection group contained 9-11 larvae.
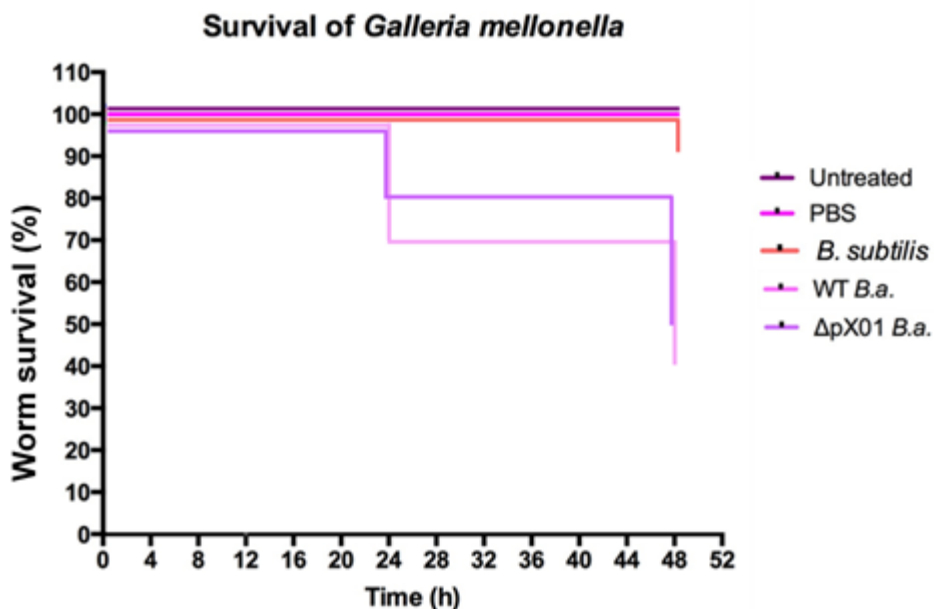
Figure 6 *G. mellonella* larvae survival rate does not correspond with relative virulence of pathogen. *G. mellonella* larvae received intrahaemocoelic injections of either WT or anthrax toxin-deficient ΔpX01 *B. anthracis* Sterne; or non-pathogenic *B. subtilis*. Injections were administered at 0 hr. Worms incubated at 37°C and percent survival was recorded ~24 and ~48 hours. Each infection group contained ten larvae.

WT *B. anthracis* Sterne and a ΔpX01 *B. anthracis* Sterne infection group using the LD_{50}

(Fig 6). *Bacillus subtilis*, a non-pathogenic species, was used as control. Because ΔpX01

B. anthracis Sterne lacks both virulence plasmids (since the Sterne strain naturally lacks

pX02), and produces neither a capsule nor the anthrax toxins, the worms inoculated with

that strain should have survival of 100%. After 48 hours, *G. mellonella* survival rates

between WT and mutant *B. anthracis* Sterne were comparable—40% survival when

treated with WT *B. anthracis* Sterne and 50% survival when treated with the virulence

plasmids double-knockout. Among worms inoculated with *B. subtilis* there was 90%

survival, indicative of the inability of the bacterium to establish infection. Our results

suggest that while *B. anthracis* Sterne can infect *G. mellonella* larvae, the waxworms

cannot be used in the context of studying variations in virulence between different strains

of the bacteria.

DISCUSSION

In this study we show that several *B. anthracis* Sterne transposon mutants displaying attenuated virulence-associated phenotypes also show evidence of disruptions in the ability to regulate oxidative stress. We identified a mutation in the gene *ywlE* that rendered the bacteria more susceptible to the reactive oxygen species H_2O_2 compared to the wild-type. In *Bacillus subtilis* under heat shock conditions, McsB targets CtsR, a repressor for heat shock genes, for degradation by ClpCP protease (19). YwlE works with the ClpC subunit of the protease to inhibit the activation of McsB, thus suppressing the heat shock response in non-stressed cells (20). Therefore, we hypothesized that a disruption in this gene in *B. anthracis* may decrease resistance to certain stresses such as exposure to ROS. However, our attempt to confirm the phenotype seen with the YwlE transposon-induced mutation by the construction of an independent insertional mutant was not successful. It is possible that an unidentified random mutation occurred in the transposon mutant resulting in the phenotype of decreased viability in H_2O_2. It is also possible that a second transposon inserted in our mutant in a different location than the one we identified, and it is the unidentified gene contributing to the increased susceptibility rather than *ywlE*. Conversely, our independent insertional mutant may not be disrupting *ywlE* as intended. Follow-up studies in which the transposon mutant ΔYwlE is complemented with a YwlE expression vector are underway to distinguish between the phenotypes of transposon mutant ΔYwlE and insertional mutant ΔYwlE.

As mentioned above, YwlE has been shown to interact with ClpC protease to suppress the heat shock response under normal conditions. Indeed, a double knockout of both YwlE and ClpC in *B. subtilis* saw the most attenuation of growth of the bacteria in

minimal media compared to a single knockout of either YwlE or ClpC (20). In *B. anthracis*, ΔClpC strains display decreased efficiency of sporulation and germination (21), indicating a role for this protease in the stress response of the bacteria. Our studies of the association of YwlE with *B. anthracis* Sterne oxidative stress response could be augmented with investigation of ΔClpC and ΔClpC ΔYwlE mutants to assess the combined effect of the two proteins on the viability of *B. anthracis* in H_2O_2.

In addition to the transposon disruption associated with YwlE, we identified eight other H_2O_2-sensitive mutants. Interestingly one of these strains possessing a mutation in a tellurium resistance gene has already been published as displaying increased susceptibility to H_2O_2 (9). We selected a second mutant, C5, whose mutation was unknown and attempted to identify the location of the transposon insertion. This particular strain displayed 14.1% growth in H_2O_2 relative to WT *B. anthracis* Sterne (Fig 1). However, efforts to sequence the amplified region were unsuccessful (data not shown). Future studies will follow up with C5 and the other eight mutants to determine their site of disruption and to confirm the genotype/phenotype association. Ultimately, we hope to continue to elucidate novel *B. anthracis* protection mechanisms against host innate immune defense such as reactive oxygen species.

In addition to using *in vitro* models of infection, such as the H_2O_2 assay, our lab is interested in using *in vivo* models to test how the presence or absence of a particular chromosome-encoded virulence factor affects the infectivity of *B. anthracis* Sterne. To do so, a relatively inexpensive and easily available model of infection must be developed. Invertebrate models are ideal because they are abundant, possess innate immune systems, and can be easily utilized for the purpose of high throughput screening. We investigated

the ability of the waxworm *G. mellonella* to be used as an invertebrate model organism

for *B. anthracis*. We found that *B. anthracis* Sterne can infect *G. mellonella* larvae and

does so in a dose-dependent manner. However, we also wanted to use *G. mellonella* as an

in vivo model for identifying novel virulence factors. Unfortunately we saw no apparent

difference in the survival rates of waxworms infected with WT *B. anthracis* Sterne and

ΔpX01, a strain of *B. anthracis* lacking both virulence plasmids and thus unable to

establish infection in most hosts. This finding renders the worms useless for our lab's

purposes. This was unexpected as differences in virulence correlating to the absence of

one virulence factor in studies of *S. aureus*-infected waxworms was reflected in

waxworm survival (13). It is still possible, however, to use the worms in studies of

antimicrobial effectiveness. For example, investigations into strains of *B. anthracis* that

display increased antibiotic sensitivity could be supplemented with the inclusion of a *G.

mellonella* infection model to which antibiotics are administered and survival is assessed.

The identification and definition of *B. anthracis* virulence factors have many

implications for human health. It allows for a broadening of the understanding of the

host-pathogen interaction, which equips scientists to better prevent and treat infection of

not only *B. anthracis* but also other clinically relevant pathogens such as methicillin-

resistant *Staphylococcus aureus*, or MRSA. With the spread of antibiotic resistant

bacteria, knowledge of a pathogen's virulence factors is necessary for the development of

anti-virulence factor therapy, an antibacterial treatment that targets the very component

needed by a pathogen to establish infection (16). Using a drug to cripple one of the

factors utilized by pathogen to invade a host results in better clearance of the bacteria by

the host immune system. Anti-virulence factor therapy has several advantages over

antibiotics including a high degree of specificity which decreases side effects, and the absence of a strong selection pressure which discourages the development of drug resistance (16). As antibiotic resistance spreads, understanding what comprises a pathogen's disease-causing arsenal will be an important area of study for the development of different modes of antibacterial therapy.

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