

ENVIRONMENTAL STRESS POTENTIATES THE INFECTIVITY OF  
BACILLUS ANTHRACIS IN CAENORHABDITIS ELEGANS

By

Vanessa A. Norris

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BACILLUS ANTHRACIS IN CAENORHABDITIS ELEGANS

Project Approved:

Phil Hartman, Ph.D.  
College of Science and Engineering  
(Supervising Professor)

Shauna McGillivray, Ph. D.  
Department of Biology

Ronald Pitcock, Ph. D.  
John V. Roach Honors College

**TABLE OF CONTENTS**

INTRODUCTION .....	1
MATERIALS AND METHODS.....	4
Solutions .....	4
Maintaining <i>C. elegans</i> Stocks.....	5
Cleaning Bacterial and Viral Contamination from Worm Stocks .....	6
Preparation of Bacteria .....	6
Dose-Dependent Infection Assay .....	7
<i>Bacillus anthracis</i> GFP Infection Assay.....	7
Sodium Chloride and Cadmium Dichloride Infection Assay .....	8
Cadmium Dichloride Titration Assay.....	8
RESULTS .....	10
Dose-Dependent Infection Assay .....	10
<i>Bacillus anthracis</i> GFP Assay .....	11
Sodium Chloride and Cadmium Dichloride Infection Assay .....	12
Cadmium Dichloride Titration Assay.....	15
TN and $\Delta$ pXO1 Mutant Infection Assay .....	16
DISCUSSION.....	17
REFERENCES .....	22
ABSTRACT.....	23

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## INTRODUCTION

Anthrax, caused by the gram-positive bacterium *Bacillus anthracis*, is a lethal, zoonotic bacterial infection resulting from exposure to bacterial endospores through cutaneous, respiratory, or gastrointestinal routes (1). Once introduced into the human body, spores are phagocytized by host macrophages, where they germinate and are transported to the lymph nodes; spores then become vegetative bacteria, leading to regional hemorrhagic lymphadenitis, or inflammation of the lymph nodes—subsequent release and replication of bacteria in the bloodstream causes severe septicemia, culminating in shock and later, death (1). Though cutaneous anthrax is most common and considered to be curable by intravenous administration of antibiotics, inhalational anthrax is the most deadly form of the infection—following the initial onset of symptoms similar to those of an upper respiratory tract infection, such as fever, cough and myalgia, death occurs within an average of three days (1). *Bacillus anthracis* is a Category A priority antigen, as designated by the National Institute of Allergy and Infectious Diseases, and due to its spore viability, rapid course of infection, and the high mortality rate of inhalational anthrax, *B. anthracis* has been used by terrorists as a bioweapon and could be potentially used in future biological warfare (2). As a result, it is highly desirable to study the bacterium in terms of its virulence and mechanism of infection in order to develop effective treatments and methods of biodefense.

Though much is understood about the pathogenesis of anthrax, the genome has not been studied extensively. The genome sequence was first analyzed and reported by Read— through his study, many important functional genes in the genome were discovered. The initiation of germination, a key initial step in infection, is facilitated by seven paralogues of the *gerA* family of tricistronic operons, and the ability of the anthrax

spore to maintain viability during dormancy is given by genes that code for DNA protection and repair proteins, including a deoxyribodipyrimidine photolyase gene specifically for repair of UV-induced DNA damage (3). Essential virulence factors and corresponding genes in the *B. anthracis* genome have also been identified. Two plasmids, pXO1 and pXO2, encode for several virulence factors and are necessary for pathogenicity. Plasmid pXO1 codes for the anthrax toxins: edema toxin and lethal toxin. Edema toxin inhibits neutrophil function, and lethal toxin induces release of tumor necrosis factor  $\alpha$  and interleukin-1 $\beta$  leading to sudden death following infection (1). Plasmid pXO2 codes for the structural genes *capB*, *capC*, and *capA* that are used in synthesis of the poly-glutamyl capsule that assists the bacteria in evasion of phagocytosis (1). In a study conducted by McGillivray et al., it was found that ClpX, a caseinolytic protease whose function is to eliminate damaged proteins, is necessary for  $\beta$ -hemolytic and proteolytic activity, resistance to killing by host antimicrobial peptides such as mammalian cathelicidins and human  $\alpha$ -defensin-2, pathogenicity and survival of vegetative bacilli *in vivo* (4). Together, these genes enable *B. anthracis* to remain dormant and germinate when conditions are optimal and also overcome host defenses in order to infect. To fully understand the manner in which these genes and their products function, it is necessary to study *B. anthracis* within live organisms.

The nematode *Caenorhabditis elegans* is a terrestrial microbivore that serves as an ideal model organism with which to investigate microbial virulence strategies in addition to how virulence factors interact with host defense mechanisms. *C. elegans* has been shown to be susceptible to several human, animal, plant and insect pathogens, including *Bacillus thuringiensis*, a member of the *Bacillus cereus* group of bacteria that is

closely related to *B. anthracis*. Susceptibility has been evidenced through the use of the worm killing process: the nematode's normal laboratory food source, *Escherichia coli* strain OP50, is replaced with the microbe of interest and worm viability and survival is closely monitored—worms fed on pathogens have a lifespan significantly shorter than the normal two weeks if fed on OP50 (5). In addition to the simplicity of the pathogenicity model, working with *C. elegans* is also advantageous because infectious microbes accumulate in the intestines of worms where ingestion can be easily visualized and the growth medium and conditions can be changed to affect virulence; furthermore, many pathogen virulence factors are conserved regardless of the host (5). The convenience with which the nematode can be used to study pathogens makes it a suitable candidate to thoroughly investigate *Bacillus anthracis*, and the possibility of genetic manipulation of both the nematode and the bacterium provides a platform for an in-depth investigation of the *C. elegans*-*B. anthracis* interaction.

*B. anthracis*, unlike *B. thuringiensis*, does not cause lethal bacterial infection to *C. elegans* (6). *B. thuringiensis* produces crystal (Cry) proteins, or pore-forming proteins (PFP), that form pores in the intestinal tract of *C. elegans* – according to a study conducted by Kho et al., the Cry5B protein accelerates lethal infection of *C. elegans* by *B. thuringiensis* in an inoculum-dependent manner and is therefore an essential virulence factor in infection. This investigation showed through Cry5B PFP-mediated assays using other *Bacillus* species that PFP increases the infectivity of the *B. cereus* group towards *C. elegans* to the highest degree, and that *B. anthracis*—which does not naturally produce crystal proteins—can be induced to infect *C. elegans* in the presence of Cry5B (6). Kho further suggests that infectivity of *B. anthracis* toward *C. elegans* is not entirely

dependent on the presence of Cry5B PFP, and could be instigated by environmental stress, starvation of nematodes, or other unidentified virulence factors.

This study focuses on the effect of different environmental stressors on the infectivity of *Bacillus anthracis* in *C. elegans*. Through manipulation of inoculum size and salt concentration in worm growth medium, it is shown that by varying the environment of growing worms, the infectivity of *Bacillus anthracis* can be potentiated in the absence of the Cry5B pore-forming protein.

## **MATERIALS AND METHODS**

### **Solutions**

#### **S-Medium**

100mL solution includes 99mL of S-basal, 100 $\mu$ L 5mg/mL cholesterol in ethanol, 1mL trace metals, 300 $\mu$ L 1M MgSO<sub>4</sub>, and 300 $\mu$ L 1M CaCl<sub>2</sub>. Cholesterol, MgSO<sub>4</sub>, and CaCl<sub>2</sub> solutions were pre-made and were provided by Dr. Phil Hartman.

#### **S-Basal**

S-Basal consists of 5.9g NaCl, 50mL KPO<sub>4</sub>, and trace metals, including EDTA, FeSO<sub>4</sub>·7H<sub>2</sub>O, ZnSO<sub>4</sub>·7H<sub>2</sub>O, MnCl<sub>2</sub>·4H<sub>2</sub>O, and CaSO<sub>4</sub>, dissolved in 1L of distilled water.

### **BHI Growth Medium**

Brain heart infusion media (BHI) was purchased from Hardy Diagnostics and

Prepared following manufacturer's instructions.

### **NaCl Stock Solution**

1.5M, 2.0M, and 2.5 M NaCl stock solutions were prepared by dissolving 8.78 g, 11.70 g, and 14.63 g NaCl, respectively, in 100mL of distilled water.

### **CdCl<sub>2</sub> Stock Solution**

1M CdCl<sub>2</sub> stock solution was prepared by dissolving 18.34 g CdCl<sub>2</sub> in 100mL of distilled water. Stock solution was diluted by a factor of 1:100 to prepare a 100mM stock solution. 100 mM CdCl<sub>2</sub> stock solution was diluted by a factor of 1:10 to prepare a 10mM stock solution.

### Maintaining *C. elegans* Stocks

*C. elegans* strain glp4 was grown in 35 mm agar plates on lawns of *Escherichia coli* strain OP50 and incubated at 16 °C. Stocks were periodically monitored to prevent overgrowth and bacterial depletion on plates. Gravid adult worms (L4) and eggs on overgrown plates were transferred to fresh plates by rinsing plates with distilled water and removing worms by sterile pipette. Worms were also transferred by sterile platinum rod. Plates contaminated by fungi were thrown out.

### Cleaning Bacterial and Viral Contamination from Worm Stocks

Adult worms were washed into 1.5mL microfuge tubes and allowed to settle; solution was then aspirated to 0.7mL of distilled H<sub>2</sub>O. Bleach (NaHClO) and 4N NaOH were then added and the solution was lab quaked for 6-8 minutes. The solution was spun to the maximum speed in a microfuge (“Steady Shake 757”) then decanted swiftly to remove excess bleach from solution; the pellet, containing nematode eggs, was resuspended in 1.0mL of sterile water and again spun to maximum speed and decanted. The pellet was resuspended in approximately 20μL of sterile H<sub>2</sub>O then plated in 3-5 drops outside a lawn of *Escherichia coli* OP50 on agar plates. Tube was tapped to completely resuspend the eggs in solution and prevent eggs from adhering to each other, which prevents egg hatching. Eggs were incubated for 72-96 hours, depending on subsequent experiment, at 16 °C. This procedure ensures that all contamination is removed from worms and that all worms are synchronized to the same developmental stage to start the infection assay.

### Preparation of Bacteria

Bacteria strains (*Escherichia coli* OP50, *Bacillus subtilis*, wild type *Bacillus anthracis* Sterne, *Bacillus anthracis* Sterne containing GFP expressed on the *pdcErm* plasmid) were inoculated in 5mL of BHI growth medium and incubated at 37 °C for 24 hrs to prepare overnight cultures for infection assays. Prior to adding bacteria to solutions for assay, cultures were spun by centrifuge (Fisher Scientific) for 10 minutes, decanted and resuspended in 5mL of distilled water. Optical density for each sample of bacteria used per trial was measured by spectrophotometer (Thermo Scientific “Genesys 20”);

optical density for each sample was contained within 5% of each other in order to control the amount of bacteria per milliliter. Bacterial samples were spun again for 10 minutes, decanted then concentrated to a final volume depending on procedure.

#### Dose-Dependent Infection Assay

*C. elegans* were grown in the presence of S-medium and either *Escherichia coli* (OP50), *Bacillus subtilis*, or *Bacillus anthracis* Sterne. First, eggs prepared by bleaching were incubated for 72 hours. Bacterial cultures were grown as described 48 hours after the onset of incubation and then concentrated to 1 mL. After the incubation period the following concentrations of bacteria were added to three 35 mm Petri dishes: 100  $\mu$ L, 200  $\mu$ L, and 400  $\mu$ L. Worms were washed off with distilled water, and added to dishes at approximately 3-4 drops per dish. Dishes were then moved to 28 °C. Infectivity of bacteria at varying concentrations was monitored by counting dead worms per plate every 48 hours for a period of 96 hours.

#### *Bacillus anthracis* GFP Assay

*C. elegans* was grown on agar plates and then exposed to either *B. subtilis*, *B. anthracis* Sterne, or *B. anthracis* GFP in 35mm Petri dishes. First, eggs prepared by bleaching were incubated for 96 hours. After 72 hours of incubation, cultures of *B. subtilis*, *B. anthracis* Sterne, and *B. anthracis* GFP were prepared and concentrated to 1.2mL. Following the incubation period, *B. subtilis* was added to Petri dishes #1-2 at concentrations of 400  $\mu$ L and 800  $\mu$ L—*B. anthracis* Sterne and *B. anthracis* GFP were added in the same concentrations to dishes #3-4 and #5-6, respectively. Incubated worms were washed off agar plates with distilled water, and added to Petri dishes at

approximately 3-4 drops per dish. Dishes were then moved to 28 °C. Infectivity of bacteria were monitored by counting dead worms per plate every 48 hours for a period of 96 hours. Plates were also observed under an inverted fluorescent microscope in order to monitor infection of *B.anthraxis* GFP.

#### Sodium Chloride and Cadmium Dichloride Titration Assay

*C. elegans* were grown in 35mm Petri dishes exposed to either S-medium only or *B. anthracis* Sterne and S-medium with the following concentrations of CdCl<sub>2</sub> and NaCl:

S-Medium Only	Plate 1: 0.0 mM CdCl <sub>2</sub> 0.0 mM NaCl	Plate 2: 10 mM CdCl <sub>2</sub>	Plate 3: 10 mM CdCl <sub>2</sub> 100 mM NaCl	Plate 4: 100 mM NaCl
<i>B. anthracis</i>	Plate 5: 0.0 mM CdCl <sub>2</sub> 0.0 mM NaCl 600 µL bacteria	Plate 6: 10 mM CdCl <sub>2</sub> 600 µL bacteria	Plate 7: 10 mM CdCl <sub>2</sub> 100 mM NaCl 600 µL bacteria	Plate 8: 100 mM NaCl 600 µL bacteria

Eggs prepared by bleaching were incubated at 16 °C for 96 hours. After 72 hours of incubation, *B. anthracis* culture was prepared overnight and concentrated to 2.4mL. Upon completion of incubation, mature worms were then washed from agar plates and added at 2-4 drops per Petri dish. Dishes were then moved to 28 °C. Infectivity of bacteria was monitored by counting dead worms per plate after 48 hours and 72 hours.

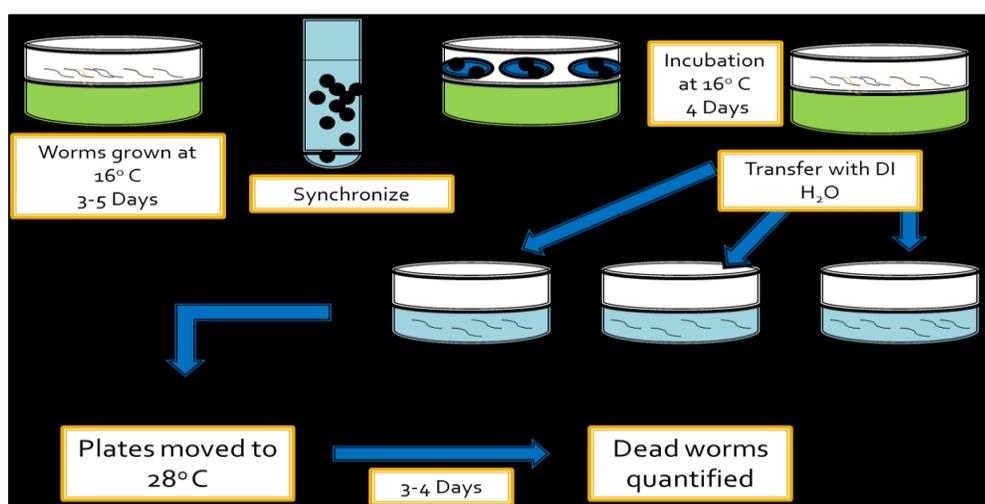
#### Cadmium Dichloride Titration Assay

*C. elegans* were grown in 35 mm Petri dishes in solution with both S-medium only, *B. subtilis* and S-medium, or *B. anthracis* Sterne and S-medium at the following concentrations of CdCl<sub>2</sub>:

S-Medium Only	Plate 1: 0.05 mM CdCl <sub>2</sub>	Plate 2: 0.1 mM CdCl <sub>2</sub>	Plate 3: 0.25 mM CdCl <sub>2</sub>	Plate 4: 0.5 mM CdCl <sub>2</sub>
<i>B. subtilis</i>	Plate 5: 0.05 mM CdCl <sub>2</sub> 600 μL <i>B.sub</i>	Plate 6: 0.1 mM CdCl <sub>2</sub> 600 μL <i>B.sub</i>	Plate 7: 0.25 mM CdCl <sub>2</sub> 600 μL <i>B.sub</i>	Plate 8: 0.5 mM CdCl <sub>2</sub> 600 μL <i>B.sub</i>
<i>B. anthracis</i>	Plate 9: 0.05 mM CdCl <sub>2</sub> 600 μL <i>B. ant</i>	Plate 10: 0.1 mM CdCl <sub>2</sub> 600 μL <i>B. ant</i>	Plate 11: 0.25 mM CdCl <sub>2</sub> 600 μL <i>B. ant</i>	Plate 12: 0.5 mM CdCl <sub>2</sub> 600 μL <i>B. ant</i>

Eggs prepared by bleaching were incubated at 16 °C for 96 hours. After 72 hours, cultures were prepared overnight and concentrated to 2.4mL. Following the incubation period, worms were transferred from agar plates to Petri dishes at 2-4 drops per plate. Dishes were then moved to 28 °C. Infectivity of bacteria was monitored by counting dead worms per plate after 48 hours and 72 hours.

The general procedure for incubation, synchronization, and worm transfer is illustrated in Figure 1.



**Figure 1. Diagram illustrates the stages of each infection assay: 1) worm growth, 2) synchronization, 3) incubation, 4) worm transfer, and 5) quantification.**

## RESULTS

### Dose-Dependent Assay

In order to qualitatively observe the susceptibility of *C. elegans* to *B. anthracis* Sterne, nematodes were first grown in the presence of either *B. anthracis* Sterne or *E. coli* OP50 as a control food source in S-medium at inoculum sizes of 100  $\mu$ L, 200  $\mu$ L and 400  $\mu$ L of bacteria. As the inoculum size of *B. anthracis* Sterne was increased, the number of deceased animals per plate increased and live animals appeared less active, as opposed to the constant growth rate and high level of activity seen in nematodes fed on OP50. These results showed that *B. anthracis* exposure alone could induce worm death, providing a platform to continue with the *B. anthracis* Sterne assays.

The dose-dependent assay was carried out in order to determine if there could be a link between the initial concentration fed to worms and the degree of lethality caused by the bacterium. Worms were fed either *B. anthracis* Sterne, *E. coli* OP50, or *Bacillus subtilis*—OP50 and *B. subtilis* are non-pathogenic and served as control treatments in the assay. Nematodes were observed over a period of 96 hours (4 days), and dead worms were quantified every 48 hours. It was found over a series of experiments that there was no noticeable pattern in the number of dead worms as bacteria concentration increased in any of the bacterial species. Also, in contrast to the hypothesis that *B. anthracis* is a more potent pathogen than OP50 and *B. subtilis*, the difference in worm death between *B. anthracis* Sterne and the control groups was inconsequential and indicated similar infectivity in *C. elegans*. However, worms grown on OP50 and *B. subtilis* grew larger

than those grown on *B. anthracis Sterne*, indicating that *B. anthracis Sterne* had some negative effect on the development of *C. elegans* starting out at the fourth larval stage.

### *B. anthracis* GFP Assay

In order to localize *B. anthracis* in the progress of infection, nematodes were fed *B. anthracis GFP* (a strain of *B. anthracis* that expressed GFP to high levels) then observed over a period of 144 hours (6 days) —dead animals per plate were counted and plates were observed under a fluorescent microscope every 48 hours. This analysis revealed an accumulation of *B. anthracis GFP* in the gut of dead and live worms, confirming that the bacterium was indeed being ingested and was causing infection. Both strains of *B. anthracis* were shown to be slightly more lethal than non-pathogenic *B. subtilis*, but as in the dose-dependent assay, the difference in worm death between species was so small it proved to be inconclusive [Table 1 and Figures 2 and 3].

**Table 1. GFP Assay. Numbers of dead worms out of total worms per plate are shown over 5 days.**

Concentration of Bacteria						
	0.4 $\mu$ L			0.8 $\mu$ L		
Species	Day 2	Day 4	Day 6	Day 2	Day 4	Day 6
<i>B. subtilis</i>	0/ 60 dead	3/60 dead	15/60 dead	0/70 dead	4/70 dead	40/70 dead
<i>B. anthracis</i>	1/70 dead	12/70 dead	20/70 dead	3/70 dead	13/70 dead	30/70 dead
<i>B. anthracis GFP</i>	2/70 dead	7/70 dead	30/70 dead	7/80 dead	17/80 dead	40/80 dead

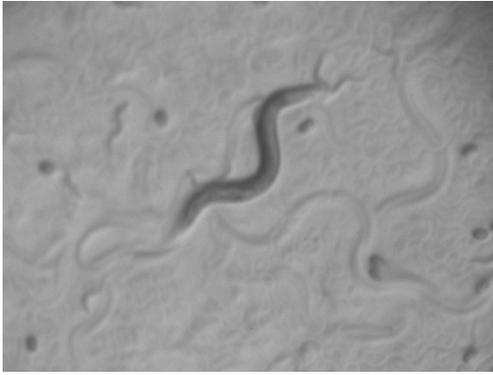


Figure 2. Live *C.elegans* after exposure to *E. coli* OP50; visualized under fluorescent microscope.



Figure 3. Dead *C.elegans* after exposure to *B. anthracis* GFP; visualized under fluorescent microscope.

### Sodium Chloride and Cadmium Chloride Infection Assay

After dose-dependency was found to be an ineffective infection paradigm, the growth medium conditions of *C. elegans* were altered in order to determine if such a change could potentiate the infectivity of *B. anthracis* Sterne. *C. elegans* was first grown in either S-medium only or *B. anthracis* Sterne, then exposed to either no salt, CdCl<sub>2</sub> alone, CdCl<sub>2</sub> and 100mM NaCl, or 100mM NaCl alone. Worms were observed over a period of 72 hours, and dead animals were quantified every 24 hours. Exposure of *C. elegans* to CdCl<sub>2</sub> – with and without NaCl – proved to be 100% lethal to nematodes within 48 hours in S-medium and *B. anthracis* Sterne; by comparison, plates not exposed to salt or exposed to NaCl only showed relatively low percentages of worm death [Table 2].

**Table 2. Sodium Chloride and Cadmium Chloride Infection Assay—Part I. Percentage of worm death per plate after 48 hours is shown.**

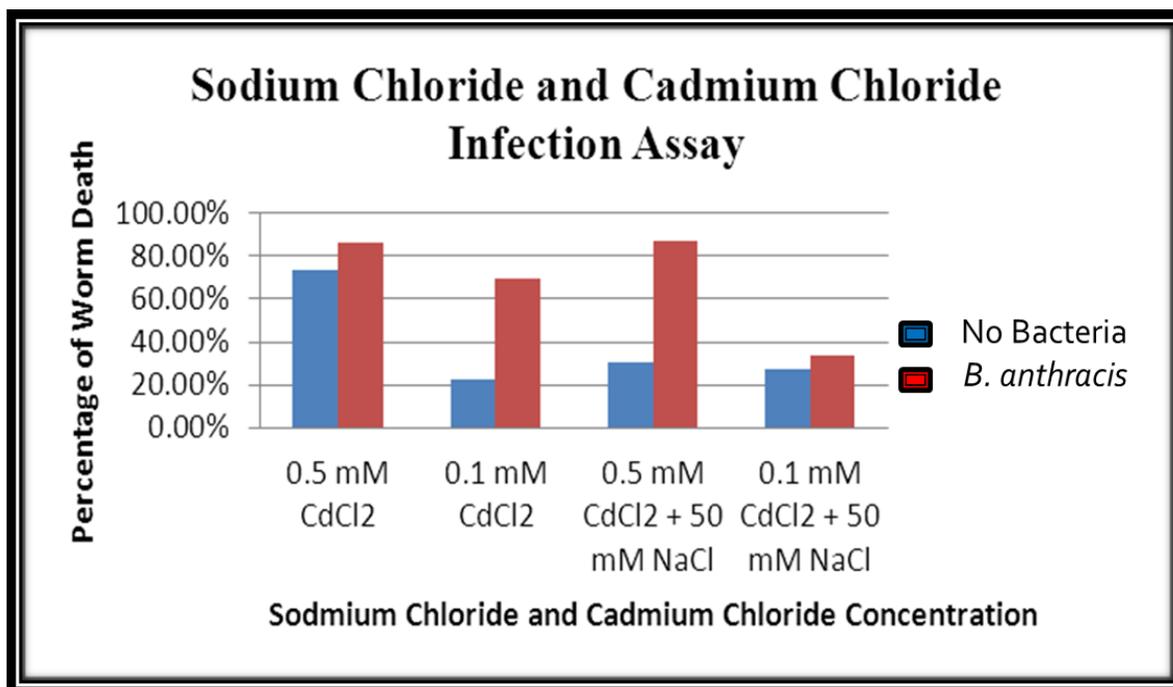
Sodium Chloride and Cadmium Chloride Concentration				
	No salt	10mM CdCl <sub>2</sub> only	10mM CdCl <sub>2</sub> + 100 mM NaCl	100 mM NaCl
Species				
S-Medium	4.0% dead	100% dead	100% dead	8.57% dead
<i>B. anthracis</i>	2.78% dead	100% dead	100% dead	3.33% dead

Exposure to cadmium chloride repeatedly led to extremely high levels of worm death. As a result, it was determined that CdCl<sub>2</sub> could be potentially used as an environmental stressor to increase *B. anthracis* infectivity. However, there was no marked difference between the percentage of worm death in *B. anthracis* and in S-medium alone—therefore, it was impossible to discern whether worm death was caused by infection or by high salt concentration. In an attempt to obtain a more clear difference in worm death between the *B. anthracis* and the control treatments, salt concentrations were decreased such that *C. elegans* was grown in the following conditions: 0.5mM CdCl<sub>2</sub>, 0.1mM CdCl<sub>2</sub>, 0.5mM CdCl<sub>2</sub> and 50mM NaCl, and 0.1mM CdCl<sub>2</sub> and 50mM NaCl. The results of this assay were highly significant: it was found that in 0.1mM CdCl<sub>2</sub> after 48 hours, there was a 3.07 fold increase in lethality of *B. anthracis* vs. S-medium only—until this assay, no conditions had been found in which *B. anthracis* was noticeably more pathogenic to *C. elegans* than other bacterial species [Table 3 and Figure 4]. The percentage of worm death in *C. elegans* grown in 0.5mM CdCl<sub>2</sub> was substantial in both S-medium and *B. anthracis*, but again, *B. anthracis* proved to be more lethal. Addition of NaCl did not result in a measureable increase or decrease in worm death. At

this stage in experimentation, a clear trend began to emerge that indicated increased pathogenicity of *B. anthracis* Sterne in conjunction with cadmium chloride.

**Table 3. Sodium Chloride and Cadmium Infection Assay—Part II. Percentage of worm death per plate after 48 hours is shown.**

	Sodium Chloride and Cadmium Chloride Concentration			
	0.5 mM CdCl <sub>2</sub>	0.1 mM CdCl <sub>2</sub>	0.5 mM CdCl <sub>2</sub> + 50 mM NaCl	0.1 mM CdCl <sub>2</sub> + 50 mM NaCl
Bacterium				
S-medium	73.33%	22.50%	30.68%	27.27%
<i>B. anthracis</i>	86.00%	69.23%	86.92%	33.57%

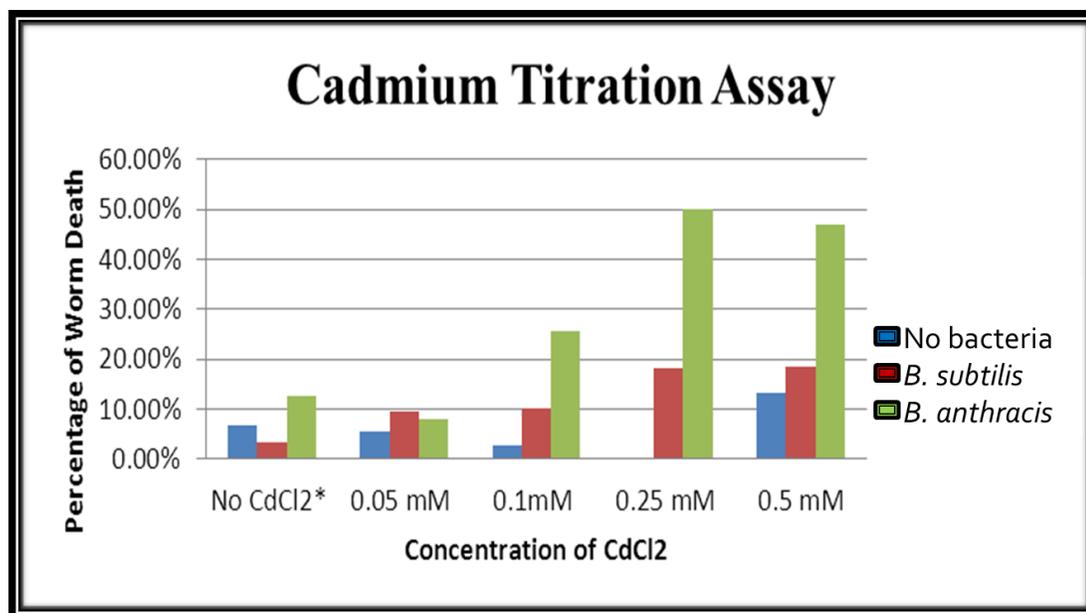


**Figure 4. Sodium Chloride and Cadmium Chloride Infection Assay. Percentage of worm death after 48 hrs shown.**

### Cadmium Chloride Titration Assay

Results of previous assays have shown that cadmium chloride exposure leads to increased worm death in *B. anthracis*. In order to determine the minimum CdCl<sub>2</sub> concentration necessary for *B. anthracis* to establish infection, *C. elegans* was grown in the presence of increasing concentrations of CdCl<sub>2</sub> in either *B. anthracis* Sterne, S-medium only or *B. subtilis* and observed over 72 hours—dead worms were counted at 48 and 72 hours. No observable pattern of increased worm death could be seen between bacterial species in 0mM CdCl<sub>2</sub> to 0.1mM CdCl<sub>2</sub> [Table 4]. However on average, *B. anthracis* was 2.64 times more lethal than *B. subtilis* in 0.25mM CdCl<sub>2</sub>, and 2.51 times more lethal than *B. subtilis* in 0.5mM CdCl<sub>2</sub> [Figure 5]. These results serve as encouraging data that identify cadmium chloride as an agent that could be used to increase the infectivity of *B. anthracis*.

<b>Table 4. Percentage of Worm Death in Cadmium Chloride Titration Assay</b>					
<b>Bacteria</b>	<b>Concentration of CdCl<sub>2</sub></b>				
	<b>No CdCl<sub>2</sub>*</b>	<b>0.05 mM</b>	<b>0.1mM</b>	<b>0.25 mM</b>	<b>0.5 mM</b>
<b>S-media</b>					
<b>24 hrs</b>	2.22%	5.56%	2.63%	0.00%	3.33%
<b>48 hrs</b>	6.60%	5.56%	2.63%	0.00%	13.33%
<b>72 hrs</b>	44.44%	33.33%	52.60%	68.18%	83.30%
<b><i>B. subtilis</i></b>					
<b>24 hrs</b>	0%	0%	7.50%	9.09%	4.65%
<b>48 hrs</b>	3.33%	9.38%	10%	18.18%	18.60%
<b>72 hrs</b>	70%	84.38%	100%	100%	100%
<b><i>B. anthracis</i></b>					
<b>24 hrs</b>	4.17%	5.26%	15.38%	15%	6.25%
<b>48 hrs</b>	12.50%	7.83%	25.64%	50%	46.88%
<b>72 hrs</b>	81.25%	92.10%	100%	100%	96.88%

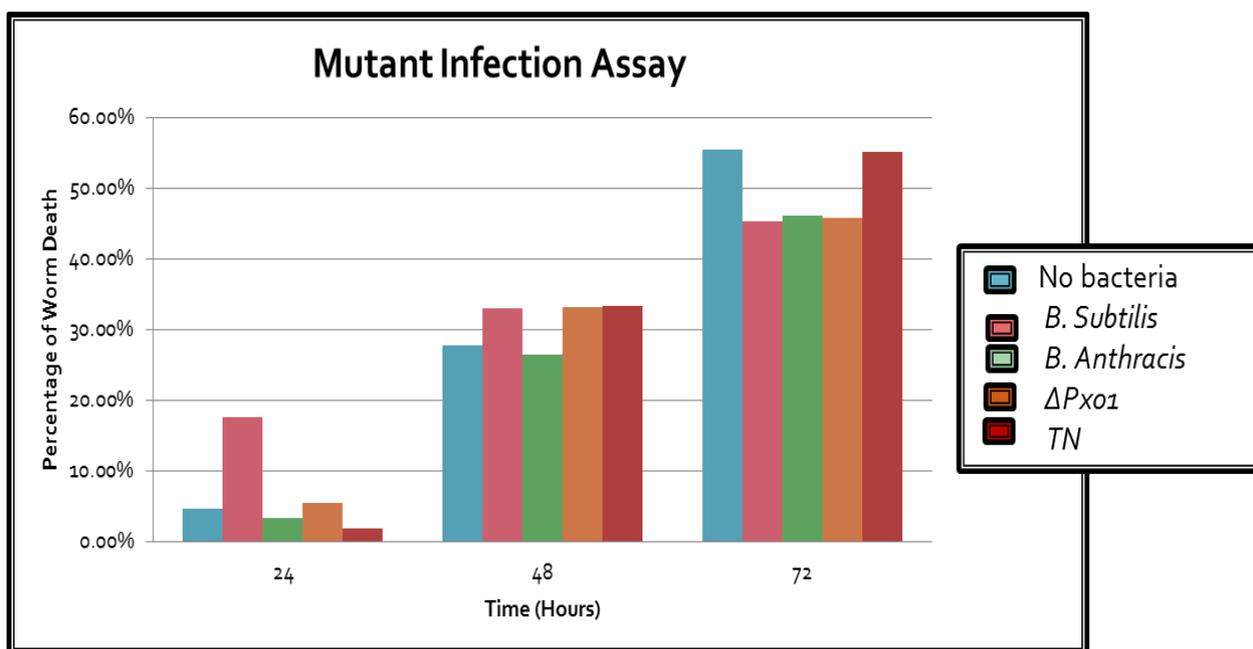


**Figure 5. Cadmium Chloride Titration Assay. Percentage of Worm Death after 48 hours is shown.**

#### TN and $\Delta$ pX01 Mutant Infection Assay

According to the pattern that had been observed in the cadmium titration assay, it was predicted that a cadmium chloride concentration of approximately 0.25mM after 48 hours would be the optimum growth medium condition for *B. anthracis* to infect *C. elegans*. This condition was applied to *B. anthracis Sterne* and two attenuated *B. anthracis* mutants: TN and  $\Delta$ pX01. The TN mutant has a transposon that interrupts the tellurium resistance operon—studies have shown that this mutant is less virulent than *B. anthracis Sterne*. The pX01 plasmid has previously been identified as a key reservoir for virulence genes; the  $\Delta$ pX01 mutant does not have this plasmid and was also expected to be less pathogenic. *C. elegans* was grown in the aforementioned condition and exposed to these strains of *B. anthracis*—it was found that *B. anthracis Sterne* was just as lethal as

the mutants, which is not consistent with previously obtained data from this study (Figure 6). Also, in contradiction with the data presented above, *B. anthracis* did not show increased pathogenicity in 0.25mM CdCl<sub>2</sub> in comparison to the control treatments (S-medium only and *B. subtilis*). Further studies must be done on the mutants in CdCl<sub>2</sub> to determine the cause of this lack of consistency in the findings.



**Figure 6. Mutant Infection Assay. Percentage of worm death after exposure to S-medium only, *B. subtilis*, *B. anthracis*, and mutants  $\Delta Px01$  and TN is shown at 24, 48 and 72 hours.**

## DISCUSSION

The Cry5B toxin has been shown to increase the pathogenicity of *B. anthracis* (6), but as this toxin is not always readily available, the discovery of various, equally effective means of pathogen potentiation would be paramount in conducting future studies on the *C. elegans*—*B. anthracis* interaction. The primary objective of this study

was to discover alternate environmental stressors by which the infectivity of *Bacillus anthracis* could be increased and reproduce a similar level of pathogenicity as with Cry5B treatment. This study investigated the effect of pathogen dosage and chloride salt concentration on pathogenicity of *B. anthracis* in the nematode *C. elegans*.

Each set of experiments provided useful feedback to aid in determination of how to structure subsequent assays, in addition to which infection paradigms required further study. The dose-dependent assay proved to be a successful tool with which to refine growth medium conditions and nematode manipulation. Though *B. anthracis* appeared to be slightly more pathogenic with increasing inoculum size than the control strains, no consistent, significant difference were observed. However, the largest difference in worm death occurred at an inoculum size of 400  $\mu$ L, indicating that *B. anthracis* was most potent at this dosage. In order to exacerbate this trend in future assays, inoculum size was increased to 600  $\mu$ L and held constant for all strains of bacteria used in the sodium chloride and cadmium dichloride titration assays. It was also found that nematodes incubated for 72 hours at 16 °C before shifting to 28 °C were extremely small and difficult to observe in solution—as a result, *C. elegans* was incubated for an additional 24 hours at 16°C before transfer to 28°C, allowing for greater nematode growth and more accurate observation.

The roughly equal magnitude of worm death in *B. anthracis*, OP50, and *B. subtilis* was initially alarming as both control strains are non-pathogenic. These findings begged the question of whether or not *B. anthracis* actually killed the nematodes as opposed to contaminated S-medium, heat or other factors. To rule out alternate possibilities, *B. anthracis GFP* was used as a visual marker of infection, S-medium was

remade, and the temperature was held constant for all plates. The presence of fluorescent material in the worms' intestines confirmed that *B. anthracis* established lethal infection in *C. elegans*.

To address the challenge of obtaining relatively equal worm death in all strains of bacteria, nematode growth conditions were manipulated to include varying concentrations of sodium chloride and cadmium chloride, exaggerating the environmental stress on the nematodes. Cadmium chloride at a concentration of 10mM was shown to be 100% lethal after 48 hours, which made it impossible to ascertain any difference between percentage of worm death in S-medium alone and *B. anthracis*. By eliminating the additional variable of NaCl, which had no significant effect, and performing a step-wise titration with dilute CdCl<sub>2</sub>, it was found that CdCl<sub>2</sub> at 0.25 mM and 0.5 mM could increase the infectivity of *B. anthracis*.

Unfortunately, cadmium chloride did not increase the percentage of worm death in *B. anthracis* Sterne when compared to the *B. anthracis* TN and ΔpXO1 mutants as predicted. When the previously observed trend was not maintained, a dilute CdCl<sub>2</sub> titration assay was conducted with fresh CdCl<sub>2</sub> in an effort to confirm increased pathogenicity at +0.25mM cadmium chloride. The pattern was also lost in this assay. At this time, it is unknown why it was no longer possible to reproduce a result in which there was selective CdCl<sub>2</sub> toxicity when infected with *B. anthracis*. In addition to cadmium chloride exposure, other variables could possibly be used to potentiate infection, including temporal temperature variation, in which nematodes are exposed to high temperature (28° C) for a short amount of time then moved back to lower temperature,

the addition of other chemicals or enzymes to the growth medium that have yet to be identified, the inhibition of *C. elegans* innate host defenses (6,7).

Discovery of a successful method of *B. anthracis* infection in *C. elegans* is a key element to the future study of *B. anthracis* subversion of the innate immune system. An understanding of *C. elegans* defenses provides a foundation for these investigations. In a study conducted by Chávez, it was found that *Caenorhabditis elegans* has two NADPH oxidases, called Ce-Duox2 and Ce-Duox1/BLI-3, encoded in its genome that produce reactive oxygen species (ROS) upon infection for host protection, much like mammalian phagocytes. Reduction of Ce-Duox1 in the intestines and hypodermis of *C. elegans* led to increased susceptibility to microbial infection (Chavez, 2009). In the *B. anthracis* transposon mutant (TN), the tellurium resistance gene is interrupted and the strain has been shown to be less virulent in wild type *C. elegans*—if this strain can be induced to infect a *C. elegans* mutant that doesn't express Ce-Duox1, a previously unidentified correlation between host ROS production and tellurium resistance could be uncovered. By taking advantage of known *C. elegans* immune defenses, it will be possible to zero in on infection mechanisms through the use of attenuated mutants such as TN.

In conclusion, *Bacillus anthracis* is a dangerous pathogen to humans and animals; as such, it is imperative to study its genome and method of infection *in vivo* in order to create specific and effective treatments. The ease with which one can expose *Caenorhabditis elegans* to the bacterium while changing growth conditions to increase pathogenicity makes the *B. anthracis*—*C. elegans* interaction a convenient model for infection. This study identified cadmium chloride as a potential trigger to induce *B.*

*anthracis* infection of *C. elegans* and could be used as a stepping stone for in-depth investigations in the future.

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## ABSTRACT

The primary objective of this study was to identify environmental stressors that could potentiate the infectivity of *Bacillus anthracis* in the nematode *Caenorhabditis elegans*. *Bacillus anthracis* is the causative agent of anthrax, a lethal bacterial infection, and has been used as a bioweapon. This pathogen must be studied in model organisms to elucidate integral steps in pathogenesis and the mechanism of infection, as well as identify bacterial virulence factors. The *B. anthracis*-*C. elegans* model system must be refined, as *B. anthracis* does not cause potent, lethal bacterial infection to *C. elegans*. However, studies have shown that *B. anthracis* can be induced to infect *C. elegans* in the presence of the Cry5B toxin, and that the infectivity of the pathogen toward *C. elegans* can be instigated by environmental stress, starvation of nematodes, etc. Through manipulation of the environment of growing *C. elegans*, I have shown that the infectivity of *Bacillus anthracis* can be potentiated in the absence of the Cry5B pore-forming protein using environmental factors.