THE ROLE OF CLPX IN

BACILLUS ANTHRACIS CELL CHARGE

by

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INTRODUCTION

Anthrax: a deadly bacterium

Anthrax is a deadly disease caused by the bacterium *Bacillus anthracis.* Endospores are the infectious agent of *B. anthracis*. They do not divide, have no measurable metabolism, and are resistant to drying, heat, ultraviolet light, gamma radiation, and many disinfectants (Watson 1994). Anthrax spores can remain dormant for decades in soil and withstand extreme environmental conditions while dormant. Their ability to be easily dispersed and remain viable indefinitely has allowed anthrax spores to be developed as biologic weapons.

Anthrax in the past

Anthrax has been circulating for centuries; Virgil described anthrax in domesticated animals in his Georgics. He even touched on its transmission to humans via wool. The Anglo-Saxons called it the black bane, the French, *charbon*; and the English, anthrax (from the Greek for coal). All of the names refer to the black pus-filled lesion that forms in cutaneous anthrax. In addition, *Bacillus anthracis* was cultured *in vitro* by Koch in 1877 and became the first bacteria to be associated with a specific disease. Koch then infected healthy animals with his culture of bacteria, thereby formulating Koch's postulates (Hart and Beeching 2002).

Anthrax today

Today, *B. anthracis* is a serious problem in parts of Africa, Spain, Greece, Turkey, Albania, Romania, central Asia, and the Middle Eastern "anthrax belt." Human cases occur most commonly in areas with domestic animals (Hart and Beeching 2002). There has never been a reported case of transmission from human-

to-human (Dixon 1999). Herbivores become infected after exposure to spores that originate in soil. Humans are infected by contact with wild animal carcasses or the meat, hides, or hair of domestic animals (Hart and Beeching 2002). Because of the deadly spores that could be contracted, it is illegal to import animal hides or wool that were not partially sterilized before shipment.

Due to its deadly nature and ease of delivery via spores, anthrax has the potential to create mass hysteria when used for bioterrorism. There has been a surge of recent interest regarding anthrax due to the 2001 terrorist attack in which letters containing anthrax spores were sent to government officials in the USA, killing 5 people and infecting 17 others. Anthrax as a weapon is actually not a new concept; during World War I, sugar cubes laced with anthrax were given to horses by Baron Otto Karl von Rosen of the German Army to disrupt transportation (Hart and Beeching, 2002). The British released anthrax spores in small bombs in 1941 and viable spores remained in the soil until the coast was sprayed with formaldehyde 45 years later. In 1993, *B. anthracis* was sprayed in downtown Tokyo by a Japanese cult. Because they mistakenly used the non-pathogenic Sterne strain on accident, they were unsuccessful in seriously harming people (Hart and Beeching, 2002).

Bacillus anthracis enters the body in three possible ways; via the skin (cutaneous anthrax), ingestion (gastrointestinal anthrax), or inhalation (inhalational anthrax) (McGillivray unpublished data). Ninety-five percent of clinical manifestations of anthrax are introduced subcutaneously through a cut; most commonly on the head, neck, and extremities (Dixon 1999). When the spores are

swallowed, the bacteria may cause lesions from the oral cavity to the cecum (Sirisanthana 2002). The deadliest and rarest form anthrax, inhalational anthrax, occurs when pathogenic endospores are breathed in and phagocytosed by alveolar macrophages (Dixon 1999.) Germination can occur at some time after inhalation; in humans up to 43 days after exposure (Meselson 1994).

Inhalational anthrax is the deadliest form of anthrax because it is most likely to become systemic and spread into the bloodstream. Systemic anthrax results when endospores enter the body by abrasion, inhalation, or ingestion and are phagocytosed by macrophages and carried to lymph nodes. Endospores germinate inside the macrophages and eventually lyse the macrophages. They replicate in the lymph nodes and spread through the blood until there are as many as one million organisms per milliliter of blood, causing severe septicemia (Dixon 1999).

Virulence factors in *Bacillus anthracis*

Anthrax bacilli express virulence factors, including two toxins and a capsule, which help them survive within the host (Dixon 1999). Protective antigen (PA), lethal factor (LF), and edema factor (EF), are encoded on the pX01 plasmid. PA is the backbone that attaches to EF or LF and facilitates entry into the cell (Brossier 2002). LF and PA together form lethal toxin, and EF together with PA forms edema toxin (ET). Edema toxin raises intracellular levels of cyclic AMP resulting in massive edema. Lethal toxin causes a hyperinflammatory condition in macrophages, resulting in reactive oxygen intermediates. LT also produces proinflammatory cytokines, such as tumor necrosis factor alpha (TNF- α) and interleukin-1 β, that can lead to shock and death (Dixon 1999). While plasmid pX01 contains the other major

pathogenicity determinants, plasmid pX02 contains the genes that code for capsule production (Brossier 2001). The capsule enables *B. anthracis* to evade complement mediated lysis and resists phagocytosis by the innate immune cells (Samant 2009). Both the toxins and the capsule are required for *B. anthracis* to be fully pathogenic (Hart and Beeching, 2002).

The Δ*clpX* mutant and cell charge

The toxins and the capsule are not the only factors that contribute to virulence. The complete genome of *B. anthracis* was sequenced in 2003, which identified 5,508 genes. A number of these chromosomal genes may contribute to pathogenesis based on homology to genes found in other pathogens (Read et al., 2003). To identify chromosomal genes linked to virulence, McGillivray et. al., applied a transposon system to generate a random mutant library of *B. anthracis*. Individual mutants were screened for loss of hemolytic activity. This revealed ClpX as the mutant that lacked both hemolytic and proteolytic activity (McGillivray et al., 2009).

To test whether clpX is necessary for virulence of *B. anthracis in vivo*, mice were infected with aerosolized spores of the Sterne strain and ΔclpX (mutant bacteria lacking a functional clpX gene) *B. anthracis.* The Sterne strain lacks a capsule and is not pathogenic in humans. 90% of the mice infected with the parental strain spores died, while all of the mice infected with ΔclpX survived (McGillivray et al., 2009). When the encapsulated Ames strain of *B. anthracis* was used, 100% of guinea pigs injected with WT died, while only 20% of guinea pigs injected with ΔclpX died (McGillivray et al., 2009). Therefore, clpX was shown to be necessary for to cause infection even in the fully virulent strain of *B. anthracis.*

ClpX functions as part of the clp protease. Caseinolytic proteases (clp) are conserved intracellular proteases that eliminate damaged, non-functional proteins and also control the lifespan of transcriptional regulators and rate-limiting enzymes (Frees 2007). clpX is responsible for recognizing, unfolding and cleaving the substrate proteins into peptides for degradation (Woo 1989). clpX also plays a role in resistance to host antimicrobial peptides and virulence in a systemic anthrax infection (McGillivray 2009). *B. anthracis* lacking clpX was more susceptible to host antimicrobial peptides as well as the antibiotic daptomycin (McGillivray 2012). AMPS are cationic proteins that kill bacteria by poking holes in the cell membrane. Daptomycin is a cationic antibiotic that targets the cell membrane and kills bacteria through depolarization of the membrane. Both AMPs and daptomycin interact with negatively charged cell surface molecules in the bacterial cell wall.

Various gram-positive bacteria possess resistance mechanisms that modify cell surface properties in order to evade degradation by cationic antimicrobial peptides. Bacteria can adjust their charge by the addition of cationic molecules, most commonly D-alanine (Samant 2009). For many gram-positive bacteria, the addition of D-alanine is encoded by the dltABCD operon (Fisher 2006 and Samant 2009). *Staphylococcus aureus, Listeria monocytogenes,* and *Streptococcus pneumoniae,* and *Bacillus anthracis* all add D-alanine through the dlt operon to increase the net positive charge on the cell surface, which repulses the cationic AMP's. The Dlt operon is essential for resistance to antimicrobial peptides as well as virulence (Samant 2009, Fisher 2006).

We hypothesized that ClpX controls cell charge through regulation of the dlt operon. For example, the clpXP protease could degrade an inhibitor of the dlt operon. The loss of clpX would then result in constant inhibition of the dlt operon, impeding its ability to contribute a positive charge. Therefore ΔclpX bacteria would have a more negative charge and increased susceptibility to cationic molecules such as AMPs and daptomycin. We investigated the difference in cell wall charge between wild type and ΔclpX mutant *B. anthracis* using two assays, Cytochrome C and Poly-L-Lysine. We also investigated whether there were differences in the expression of the dlt operon between WT and ΔclpX bacteria.

MATERIALS AND METHODS

Growth Conditions and Bacterial Strains

Bacillus anthracis Sterne strain ($pXO1^+$ $pXO2^-$) and isogenic mutant bacteria lacking *clpX* (ΔclpX) were used (McGillivray 2009). The bacteria were grown in Brain-Heart Infusion (BHI) medium at 37°C in a shaking incubator.

Cell Charge Assays

Cytochrome C

Overnight cultures of WT *B. anthracis* and ΔclpX *B. anthracis* mutant were grown in 2ml of BHI. 500 µl of the overnight ΔclpX culture was added to 10 ml of BHI in a glass tube and 350 µl of WT culture was added to 10 ml of BHI in a glass tube. The cultures were grown in a shaker at 37°C for 2-4 hours until the bacteria reached early log phase (optical density of 0.4). Once the WT and ΔclpX reached log phase, the tubes were centrifuged at 4000 rpm for 5 minutes. The pellets were washed with 1 ml of 20 mM MOPS, pH7 and centrifuged at 4000 rpm for 5 minutes.

300 µl of 20 mM MOPS was added to each pellet and resuspended. The bacteria sample was diluted 1:20 using 20 mM MOPS in wells on a plate, then the OD600 was read using a plate reader. The volume of bacteria needed to give a final optical density of 1.0 in 250µl total was determined using the equation, $OD_{\text{diluted bacteria}}$ x 20 (dilution factor) = $OD_{\text{undiluted bacteria}}$. The volume of undiluted bacteria was found using the equation ($OD600_{\text{undiluted bacteria}}$) x (N µl) = 1 x 250 µl. The amount of MOPS to add was calculated by subtracting the amount of undiluted bacteria to add and the amount of cytochrome C to add (25 µl) from the total volume of 250 µl. The calculated amounts of undiluted bacteria, MOPs buffer, and cytochrome C were added to a 1.5 ml tube (WT and Δ*clpX* in separate tubes), vortexed, and incubated at room temperature for 30 minutes. The tubes were then centrifuged at max speed for 5 minutes. 200 µl of supernatant was transferred to individual wells of a 96 well plate and the OD530 was recorded.

*Various conditions were implemented; sometimes doubling the bacteria to Cytochrome C ratio, other attempts combining to make 3x, 4x, 5x, 6x, 7x, 8x and 10x the total volume with the original conditions.

Poly-l-lysine

Overnight cultures of WT *B. anthracis* and ΔclpX *B. anthracis* mutant were grown in 2ml of BHI. Bacteria were used in either in stationary (overnight cultures) or log phase (as described above). The tubes were centrifuged at 4000 rpm for 5 minutes. 300 µl of 20 mM HEPES buffer, pH 7.5, was added to each pellet and resuspended. The bacteria sample was diluted 1:20 using 20 mM HEPES in wells on

a plate, then the OD600 was read using a plate reader. The volume of bacteria needed to give an optical density of 0.1 and 1.0. 250µl total was determined using the equation, $OD_{\text{diluted bacteria}}$ x 20 (dilution factor) = $OD_{\text{undiluted bacteria}}$. The volume of undiluted bacteria was found using the equation ($OD600_{\text{undiluted bacteria}}$) x (N µl) = 1 x 250 µl. The amount of HEPES to add was calculated by subtracting the amount of undiluted bacteria to add and the amount of poly-l-lysine to add (2.5 µl) from the total volume of 250 µl. The calculated amounts of undiluted bacteria, HEPES buffer, and Poly-l-lysine were added to a 1.5 ml tube (WT and ΔclpX in separate tubes), vortexed, and incubated at room temperature for 30 minutes. The tubes were then centrifuged at max speed for 5 minutes. 200 µl of supernatant was transferred to individual wells of a 96 well plate and the fluorescence 485 was recorded.

QPCR

8 µl water, 10 µl Sybr Green (Invitrogen), and .5 µl Fus A fwd (10 µM) and .5 µl Fus A (10 µM) reverse short were combined to create a Fus A master mix. 8 µl Water, 10 µl Syber Green, .5 µl Dlt Forward and .5 µl Dlt reverse short were combined to create a separate Dlt master mix. This was multiplied by the number of wells in the 96 well plate that were to be used. 19 µl of the master mix was added to each well on the plate. 1µl of cDNA from either WT or ΔclpX log-phase bacteria was added to each well to constitute a 20 µl total. All conditions were performed in triplicate in order to obtain more standardized results.

RESULTS

Cell charge is slightly decreased in Δ*clpX*

Deletion of clpX in *Bacillus anthracis* results in decreased defense against cationic antimicrobial peptides (McGillivray et al., 2009). A known defense against cationic AMPs is making the bacterial cell wall more positive to repel them (Samant et al., 2009) We hypothesized that the loss of clpX induces a net negative charge, which makes the mutant bacteria more susceptible to AMPs and thus contributes to the decrease in virulence. Two assays were performed to determine cell charge differences between WT *B. anthracis* and the clpX mutant. Two positively charged molecules, Cytochrome C, which is red in color, and Poly-L-Lysine, which has a fluorescent tag, should interact with the negatively charged ΔclpX mutant more than the WT bacterial pellet. The supernatant from the ΔclpX mutant incubated with Cytochrome C is expected to have a lower optical density (lighter supernatant) because the positively charged molecules remain bonded to the bacterial pellet. The Δ*clpX* mutant incubated with Poly-L-lysine should have less fluorescence in the supernatant because the positively charged dye is expected to remain attached to the bacterial pellet.

Cell charge measured using Cytochrome C

We first assessed cell charge using the Cytochrome C assay. *Streptococcus pyogenes* was used as a positive control because it has been previously proven that the Δdlt mutant is indeed more negatively charged (Samant 2009.) As can be seen in Figure 1.2, less cytochrome C was observed in the supernatant of Δdlt *Streptococcus pyogenes,* therefore verifying our assay conditions. In *B. anthracis* after incubation

with Cytochrome C, we find that WT had an OD600 of 0.498 and that of the Δ*clpX* mutant was 0.386 (Fig. 1.1). This difference in OD readings of the supernatant between WT and ΔclpX was not statistically significant. If ΔclpX were more negatively charged, we would also predict a visible difference in pellet color between WT and ΔclpX. We did see a slightly darker pellet in ΔclpX (Fig. 1.3) indicating there might a slight difference in cell charge. However, although there may be a trend, we cannot conclude that Δclp*X* is more negatively charged in comparison to WT.

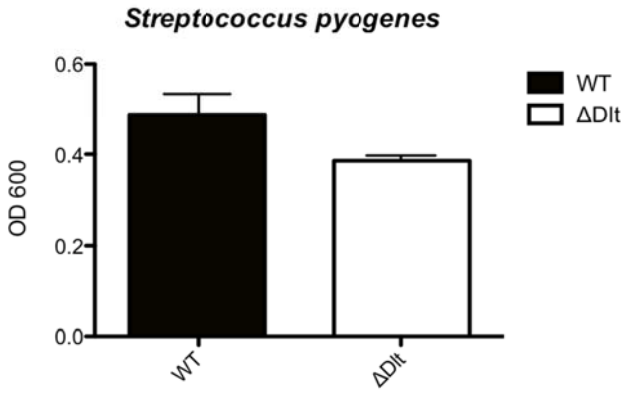

Figure 1.1 **Binding of postively charged Cytochrome C to WT and ΔDlt *Streptococcus pyogenes*.** The optical density at 600nm of the supernatant after a 30 minute incubation of *Streptococcus pyogenes* with cytochrome C with a concentration of 1.0 mg/ml. *, p<0.05 by student's t-test

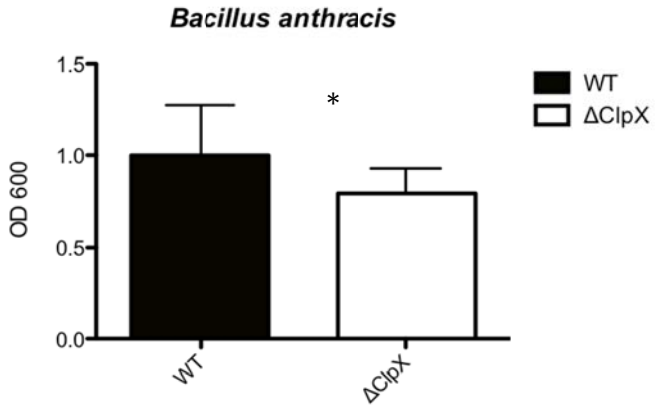

Figure 1.2 **Binding of positively charged Cytochrome C to WT and ΔClpX *Bacillus anthracis.*** The optical density at 600nm of the supernatant after a 30 minute incubation of *Bacillus anthracis* with cytochrome C with a concentration of 1.0 mg/ml.

Various conditions were tried for the Cytochrome C assay in order to optimize assay conditions. For example, log phase cultures (overnight) were substituted for stationary phase cultures, the concentration of Cytochrome C was doubled, and different total volumes were used. However, no significant difference was seen using different assay conditions.

Cell charge measured using Poly-l-lysine

We next tested cellular charge by incubating the bacteria with a different positively charged molecule, poly-l-lysine that is fluorescently labeled, to make sure our results were not just specific for Cytochrome C. We noted varied results in which certain trials yielded drastic differences in fluorescence between WT and ΔclpX yet other trials produced conflicting data or no variation in WT or ΔclpX whatsoever. The *Streptococcus pyogenes* control also did not yield any significant difference between WT and ΔclpX cell charge (Fig 2.2). Therefore it is difficult to

draw any conclusions from the Poly-l-lysine results in *B. anthracis* because there was not a functional control.

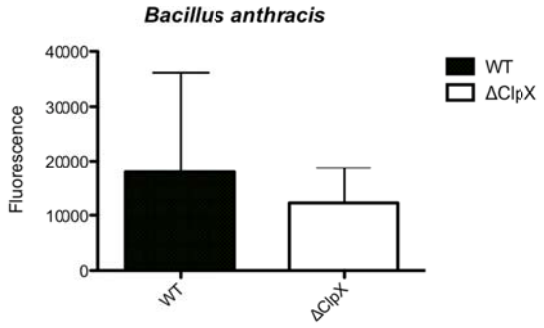

Figure 2.1 **Binding of postively charged Poly-L-lysine to WT and ΔClpX *Bacillus anthracis*.**
The flucrescence at 485 nm of the supernatant after a 30 minute incubation of *Bacillus anthracis* with Poly-L-lysine with a concentration of 10µg/ml.

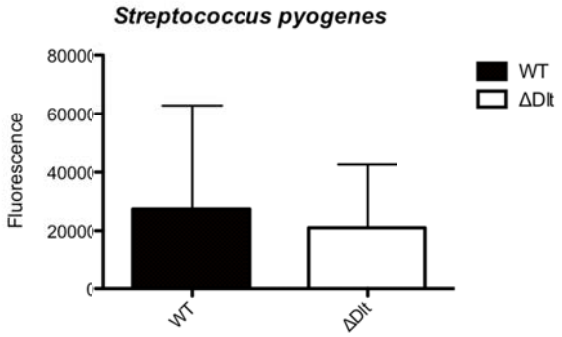

Figure 2.2 **Binding of postively charged Poly-L-lysine to WT and ΔDlt *Streptococcus pyogenes***
The fluorescence at 485 nm of the supernatant after a 30 minute incubation of *Strep. pyogenes* with Poly-L-lysine with a concentration of 10µg/ml.

The dlt operon

The dlt operon is known to induce a positive charge in various bacteria species in order to repel host AMP's (Samant 2009, Fisher 2006.) In order to test whether clpX is necessary for proper regulation of the dlt operon, we compared dlt expression in WT and ΔclpX *B. anthracis* by using QPCR. QPCR is a process in which

one specific gene is amplified (in this case, the first gene in the dlt operon) and gene expression can be quantified. We found that the ratio of WT to ΔclpX expression was 1:1.36 (Fig. 3.1). This difference is not statistically significant and therefore we conclude that there is likely little to no difference in the regulation of the dlt operon between WT and ΔclpX *B. anthracis*.

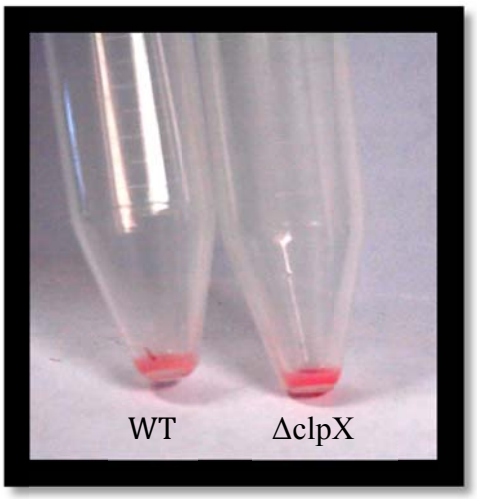

Figure 1.3 **Pellet color in WT vs ΔclpX in *Bacillus anthracis*.** The supernatant was discarded after a 30 minute incubation of *Bacillus anthracis* with cytochrome C with a concentration of 1.0 mg/ml. Note the brighter red color in the ΔClpX pellet.

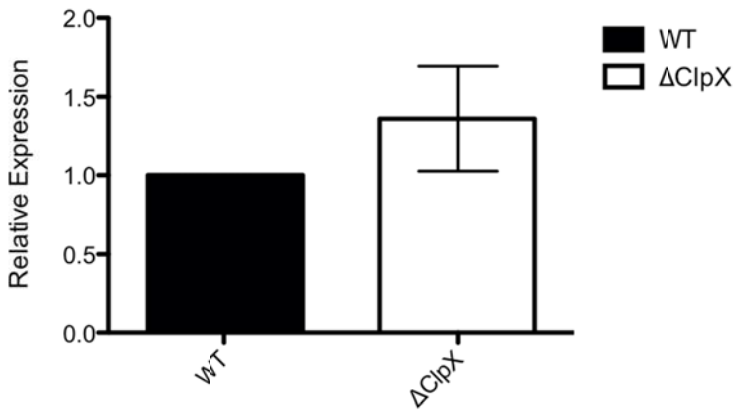

Figure 3.1 **Expression of Dlt operon in WT and ΔClpX *Bacillus anthracis***
Expression of dltA normalized to FusA in cDNA extracted from log phase WT and ΔClpX bacteria.

DISCUSSION

Lack of statistically significant data

Loss of clpX in *Bacillus anthracis* has been shown to significantly decrease virulence (McGillivray 2009). We investigated the hypothesis that the ΔclpX mutant is more susceptible to antimicrobial peptides of the host because of differences in cell wall charge between WT and ΔclpX. Cellular charge was assayed using cytochrome C and Poly-L-lysine assays. These assays measured how well the positively charged cytochrome C and Poly-L-Lysine bound to the bacterial pellet by quantifying the amount of color or fluorescence that remained in the supernatant. Although there was a noticeable difference in pellet color, the cytochrome C assay did not yield statistically significant results from the OD readings of the supernatant. The poly-l-lysine assay similarly did not produce significantly different data. However, the poly-l-lysine assay also did not work with our positive control suggesting that assay conditions were not optimized.

Lack of a true control

In addition, both assays lacked a *true* control. *Streptococcus pyogenes* ΔdltD was used as the control, however a more accurate control would compare *Bacillus anthracis* ΔdltD to *Bacillus anthracis* ΔclpX. This would be a better control because we would be comparing the same species of bacteria rather than two different species. Thus, before any definitive conclusions are drawn, a *Bacillus anthracis* ΔdltD should be obtained to use as the control.

We also used QPCR to investigate differences in dlt operon expression between WT and ΔclpX *B. anthracis.* Our results revealed that the difference in expression of the dlt operon in WT and ΔclpX is likely negligible. Taken together, our results likely indicate that clpX does not alter cell charge and our hypothesis is incorrect.

Other possible factors influencing ΔClpX susceptibility

The cell charge of ΔclpX is not the only component that is suspected to aid in the survival of ΔclpX *Bacillus anthracis*. Another student in our lab is currently conducting experiments on autolysis and cell wall thickness which also may be responsible for the increased susceptibility of ΔclpX to host antimicrobial peptides. Autolysis is a natural process in which the bacteria break down the peptidoglycan of its cell wall in order to expand and grow in response to variable environments. Cell wall autolysis makes the bacteria more susceptible to antimicrobial peptides and antibiotics because of its weaker cell wall (Groicher 2000, Boyle- Vavra 2003). We are currently investigating the difference in autolytic activity between WT and ΔclpX *B. anthracis*. We are also employing QPCR to investigate the expression of genes

known to regulate autolysis. Cell wall thickness analysis reveals that a thicker cell wall is harder for the antimicrobial peptides or antibiotics to penetrate (Cui 2006). We are using an electron microscope to determine if the cell wall of ΔclpX is thinner and therefore more susceptible to antibiotics.

The loss of clpX has been shown to contribute to *Bacillus anthracis*' susceptibility to the antimicrobial peptides of the host immune system and to cell-wall acting antibiotics (McGillivray 2009). Our tests attempted to determine what specific factors contribute to the bacterial susceptibility of ΔclpX. Our initial hypothesis was not proven to be correct, and cell charge is probably not be the reason for increased susceptibility. However, other factors including autolysis and cell thickness may be affected by loss of clpX and future efforts will investigate those factors. The reasons why clpX leads to decreased pathogenicity are still unclear and more research is required to solidify our understanding of the role clpX in the cell. ClpXP is an intriguing pharmacological target because, if it is inhibited, then it may decrease the pathogenicity of the bacteria in the host. When the clpXP is inactivated, the bacteria will be more susceptible to antibiotics and the host defenses may be eliminate the bacteria from the host with greater success. Therefore, future investigations of the clpXP protease are warranted.

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ABSTRACT

Anthrax is a deadly disease caused by the gram-positive bacterium *Bacillus anthracis.* In order to be fully virulent, the bacteria must exhibit lethal factor toxin, edema toxin, protective antigen, and a capsule. We have previously shown that another virulence factor is clpXP, an intracellular protease that degrades specific proteins. The loss of clpX in *B. anthracis* led to a decrease in pathogenesis and increase in antibiotic and antimicrobial peptide susceptibility. It has been shown in *Streptococcus pneumoniae* that a more positively charged cell wall has lead to increased antimicrobial peptide resistance. Due to the natural positive charge of antimicrobial peptides of the innate immune system, they are thought to be repelled by a positively charged cell wall. We hypothesize that the loss of clpX induces a net negative charge, which makes the mutant bacteria more susceptible to AMPs and thus contributes to the decrease in virulence. We also investigated the regulation of the dlt operon which is known to control cell charge in other bacteria species. We used Cytochrome C, a positively charged red dye, and Poly-L-lysine, a positively charged fluorescent dye, to compare the cell charge of the clpX mutant and its parent strain.