ROLE OF THE CLPATPASE FAMILY MEMBERS IN THE STRESS RESPONSE OF *BACILLUS ANTHRACIS*

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Introduction

Anthrax is a serious infectious disease caused by the bacterial pathogen *Bacillus anthracis*, which is a gram-positive, rod-shaped, spore forming bacterium. *Bacillus anthracis* has two different life cycles depending upon the environment, which is either in the soil or in the mammalian host [1]. In the soil, the bacterium undergoes a cycle of saprophytic growth, sporulation, and germination. In contrast, the life cycle of *B. anthracis* in the mammalian host is spore-germination, which is followed by the replication of the vegetative cell [1]. Once endospores of *B. anthracis* enter the human body, host macrophages carry and release them into the lymph nodes where they germinate and multiply [2]. High numbers of bacteria spread throughout the body via the blood and lymph, causing severe septicemia. Death resulting from septicemia and other complications occurs approximately seven days after exposure [2].

Anthrax is a zoonotic disease and occurs mainly in grazing animals. Humans catch the disease through exposure with infected animal or animal products. There are no recorded cases of human-human transmission [3]. There are 3 different kinds of anthrax infection depending upon the route of entry, including cutaneous infection, gastrointestinal infection, and inhalational infection. The endospore can enter the skin through abrasions or cuts resulting in cutaneous infection, which accounts for 95% of human cases world-wide [4]. One of the hallmark symptoms for cutaneous infection is a cluster of boils, which appears black in the center surrounded by a bright red lesion [4]. In gastrointestinal infection, endospores enter via ingesting contaminated food that leads to ulceration in the mouth or stomach causing abdominal pain. Inhalational infection is the least common type of anthrax, accounting for only about 5% of human cases but it has the highest mortality rate. When the endospores enter through inhalation, it can trigger severe bacteremia and toxemia via a systemic infection that is often fatal [3].

Bacillus anthracis is categorized as a Category A Priority Pathogen by the National Institute of Allergy and Infectious Diseases, indicating that it poses the highest danger to national security and public health owing to its ease of transmission and severity of sickness. *Bacillus anthracis* has been used as a bioterror weapon in the past, most notably in 2001, when endospores were sent via the US mail, resulting in 18 confirmed cases of anthrax with five deaths [2]. Our lab has been studying the virulence of *B. anthracis* to gain a better understanding of how this pathogen is so adept at evading host defenses resulting in severe infection.

The high virulence of *B. anthracis* is due to numerous virulence genes encoded in the genome of the bacteria, which consists of around 5000 chromosomal genes and two plasmids, pXO1 and pXO2 [2, 3]. Many studies have demonstrated that the *B. anthracis* plasmids are necessary for virulence. pXO1 encodes the genes for lethal factor, edema factor and protective antigen. Both lethal and edema factors bind to the protective antigen forming the lethal and edema toxins, which interfere with cellular signaling of host immune response cells [5-7]. pXO2 encodes the genes necessary for the capsule, which is responsible for protecting the bacteria from host phagocytosis [3]. Full virulence in *B. anthracis* requires the presence of both plasmids [2, 4, 7]. The role of chromosomal genes in virulence has not been as well studied. Recently, evidence from several labs including our own demonstrate that chromosomal genes also play an important role in the virulence [6]. The whole chromosomal sequence of *B. anthracis* Ames was analyzed to find additional genes that are important for virulence and some chromosomally encoded proteins, including phospholipases, and iron acquisition activities, were identified as important targets for therapeutics [8]. Studying virulence helps us to gain a better understanding of the pathogenesis of *B. anthracis* and identify future potential antibiotic targets that could help fight infection.

Our lab used a transposon-based mutagenesis system, which allows for random transposon insertion and disruption of chromosomal genes, to construct a library of *B. anthracis* mutants, each theoretically with a different interrupted gene [6]. We were successful in employing this system to identify the novel role of the *yceGH* operon, which contributes to virulence in *B. anthracis* through defense against reactive oxygen species [9]. Using the same system, we also identified the *clpX* gene as playing a critical role in virulence of *B. anthracis* through defense against host antimicrobial peptides [6, 10]. Later discoveries demonstrated that loss of *clpX* increases susceptibility to primarily cell-envelope active antibiotics such as penicillin and daptomycin [10]. The cell envelope of a bacterium consists of the cell wall and cell membrane. Penicillin interferes with activity of penicillin-binding proteins (also known as transpeptidases), blocking the construction of the cell wall [11]. Daptomycin causes depolarization and permeabilization of the bacterial cell membrane, although it also interacts with the cell wall and modifications of the cell wall are known to alter daptomycin resistance [12]. More recently, we have demonstrated that loss of *clpX* results in changes to cell wall morphology in *B. anthracis* Sterne [13].

ClpX is one of several Clp ATPases that interacts with a proteolytic core called ClpP to form the Clp protease complex, ClpXP [14]. Beside protease activity, Clp ATPases can function independently as a chaperone where it takes misfolded proteins and converts them back to the correctly folded protein. Clp ATPases are highly conserved in many bacteria, and they have been linked to virulence and the stress response in several pathogens, especially gram-positive bacteria [14, 15]. ClpP possesses protease function, but by itself is only degrades small peptides. However, ClpP can cooperate with Clp ATPases such as ClpX to degrade large proteins. ClpX recognizes the protein substrates and utilizes the energy generated during ATP hydrolysis to generate a pulling force that unravels folded protein domains in preparation for translocation into

the ClpP degradation chamber [13]. ClpXP degrades a variety of targets, including rate-limiting enzymes, stress response proteins, and transcriptional regulators, and hence acts as a global regulator [14, 15]. Therefore, loss of the ClpXP protease, either through *clpX* or *clpP* gene deletion, will have pleotropic effects on the bacterial cell [15]. Notably, *B. anthracis* has two ClpP subunits, *clpP1* and *clpP2* [13]. *Mycobacterium tuberculosis* and gram-negative *Pseudomonas aeruginosa* contain two ClpP subunits, but this is relatively rare among gram-positive bacteria [16, 17]. How ClpP1 and ClpP2 cooperate with each other and with ClpX in *B. anthracis* remains unclear; however, they were found to be important for host cell-envelope antibiotic resistance in *B. anthracis* [13].

While we have shown that ClpXP plays a role in resistance to antibiotic stress, there are also other stress responses such as temperature, acid, salt, or oxidative stress that are linked to the Clp system [14, 15, 18]. Loss of *clpX* leads to reduced growth under acidic pH and *clpX* is required for growth during cold stress in *Streptococcus suis* [19]. Moreover, the inactivation of *clpX* also affects growth under heat stress (50^{o}C) or salt stress in *Bacillus subtilis* [20]. Based on previous studies, our first aim is to investigate if *clpX* is responsible for regulation in other stress responses beside antibiotic resistance. Moreover, if *clpX* is important for other stress responses, then we want to determine whether it is mediated through its chaperone activity independently or its protease activity in conjunction with ClpP.

Besides ClpX, there are other Clp ATPase family members such as ClpB and ClpC, whose role in *B. anthracis* has remained largely unexplored when trying to understand the stress response including antibiotic resistance. Similar to ClpX, ClpC possesses a dual mechanism so that it can act independently as a chaperone or cooperate with ClpP to form the ClpCP protease complex [14]. However, ClpC is structurally distinct from ClpX and contains a distinct group of

adaptor proteins; thus, the spectrum of regulated proteins for ClpCP is distinct from that of ClpXP, suggesting that it may perform a different regulatory role than ClpXP. ClpC is required for growth during heat stress (43^{o}C) and ClpC plays an essential role in viable spore formation and germination in *B. anthracis*, specifically at engulfment stage; however, its role in other stress responses such as antibiotic resistance or virulence has not yet been explored in *B. anthraci*s [21, 22]. ClpC also has been connected to virulence in several other pathogens as well [23-25]. Loss of ClpC leads to increased susceptibility to oxidative stress and heat stress in both *S. aureus* and *Listeria monocytogenes* [23, 26]. In *S. aureus*, ClpC is important for allowing the formation of persister cells, which persist in a non-growing state allowing them to survive in the presence of antibiotics, specifically oxacillin [27].

Unlike ClpX and ClpC, which have multiple functions, ClpB does not collaborate with a peptidase partner but acts alone as a remodeling enzyme. To disaggregate stress-denatured proteins, ClpB works together with the DnaK system, a fundamental component of the cellular network of molecular chaperones and folding catalysts [28]. ClpB is highly conserved in bacteria, fungi, protozoa, and plants, and its function under a variety of stressful situations has been extensively investigated [29]. It protects against, among other things, heat, low pH, osmotic- and oxidative stress, ethanol, and nutritional deprivation [30-32]. ClpB is an essential stress regulator of *M. tuberculosis* against heat stress, oxidative stress, or a nutrient-deficient environment [32]. ClpB has been linked to virulence in *Leptospira interrogans* and *L. monocytogenes* [33, 34]. It also plays a critical role in thermotolerance allowing *L. monocytogenes* to survive in high temperatures [34]. Loss of *clpB* leads to an increased susceptibility in heat stress or oxidative stress in *Salmonella typhimurium* [35]. In addition, ClpB has been demonstrated to affect the expression of virulence proteins in many pathogenic bacteria including *L. monocytogenes, L. interrogans* and *S. typhimurium* [18, 36, 37]. However, the function of ClpB in *B. anthracis*

remains unclear. Therefore, our second aim is to investigate the roles of ClpB and ClpC in regulating not only antibiotic resistance but also other stress responses in *B. anthracis*. Gaining a better understanding of the role of ClpATPase family members in regulation of multiple stress responses could provide us more targets to either directly fight infection or to help make current antibiotics more effective.

Methods

Bacterial strains and culture

Bacillus anthracis Sterne ($pXO1^+$, $pXO2^-$) was used as the parental strain in this study. *B.anthracis ΔclpX* was previously described as were the wild-type parental *B. anthracis* Sterne carrying the empty inducible plasmid pUTE657, the isogenic *ΔclpX* mutant retaining empty *p*UTE657, and the *ΔclpX* mutant supplemented with the wild-type *clpX* gene in pUTE657 (*pclpX*) [38]. The *ΔclpX +pclpX*^{I265E} mutant, as well as the two *clpP* insertional mutants, *ΔclpP1* and *ΔclpP2*, were also constructed previously [13]. Construction of additional mutants is detailed below. *Bacillus anthracis* Sterne was grown aerobically in brain heart infusion (BHI) at 37°C shaking. To induce the expression plasmid, 0.1 mM isopropyl β-d-1thiogalactopyranoside (IPTG) was added.

Construction of *B. anthracis clpB* and *clpC* insertional mutants

We amplified approximately 500 bp of DNA in the middle of the *clpB* gene with primers ClpB IM-2 Fwd-XhoI (5'- ACA GTC TCG AGC TTT GCG TTA GAT ATG AGT GCG CT – 3') and ClpB IM-2 Rev-HINDIII (5' – GAC TAA GCT TCA CGC TTC GTC AAC AAG ATC -3'). We amplified 456 bp of DNA in the middle of the *clpC* gene with primers ClpC IM Fwd-EcoRI (5' – AGT GAA TTC TGG TTT AAT CCG CGA AGG TG – 3') and ClpC IM Rev-HINDIII (5'

ACT AAG CTT CCA CGG TAT TTC GTT CCA GC – 3'). The resultant amplicon was subcloned into the temperature-sensitive plasmid pHY304 using the restriction endonucleases XhoI and HINDIII for *clpB* and EcoRI and HINDIII for *clpC*. We transformed these plasmids containing the resultant amplicon first into electrocompetent MC1061F *Escherichia coli* cells (Lucigen) and subsequently into the methylation-deficient *E. coli* strain GM2163. We used PCR and gel electrophoresis on each transformation to validate the success of the plasmid transformation, followed by purification before doing another transformation. Plasmid from GM2163 cells was purified and transformed into *B. anthracis* Sterne. We validated these transformants using primers pHY3065 Fwd (5' ACG ACT CAC TAT AGG GCG AAT TGG – 3') and pHY3175 Rev (5' CCG CTC TAG AAC TAG TGG ATC CCC – 3'). We cultured *B. anthracis* Sterne with mutagenic plasmid overnight at 30^{o}C shaking in BHI containing 5 μg/ml erythromycin. We cultured 100μL of each overnight culture in fresh BHI with 5 μg/ml erythromycin at 37^{o}C shaking for at least 8 hours to allow the plasmid to incorporate into the genome. The *clpB* insertional mutant in *B. anthracis* was validated using the primers pHY3065 Fwd (5' ACG ACT CAC TAT AGG GCG AAT TGG – 3') and ClpB IM-2 confirm Rev (5' CTC CCA TTT CGC TCT CAT GC – 3'), and the *clpC* insertional mutant in *B. anthracis* was verified using the primers pHY3065 Fwd (5' ACG ACT CAC TAT AGG GCG AAT TGG – 3') and ClpC IM confirm Rev-2 (5' CAA CGT GAA TTG GTT GGA AAC GTC – 3').

Acid stress assay

Overnight cultures were grown in BHI at 37°C shaking and supplemented with antibiotics as specified. Then, we grew to log phase at an optical density (OD) of 0.4-0.5 at a 600 nm wavelength in fresh BHI at 37°C shaking. We collected 1 mL of each log phase culture, pelleted it, washed it, and resuspended it in an equal amount of PBS with a pH of 7 or 4. Following a

second wash, the cultures were diluted 1:2 in PBS and left on the bench at room temperature. The corresponding 1:2 dilution was also used to complete a 1:10 serial dilution, and the 1:20, 1:200, and 1:2000 dilutions were plated on BHI every two hours for a minimum of four hours to determine the approximate cfu of culture. The plates were incubated at 30°C for 16-18 hours before colonies were counted. When assessing strains with expression plasmid, we added 0.1 mM IPTG to overnight and log phase cultures, but no additional IPTG was added to PBS when doing the survival assay.

Temperature stress assay

Overnight cultures were grown in BHI at 37°C shaking and supplemented with antibiotics as specified. Then, we grew to log phase at an optical density (OD) of 0.4-0.5 at a 600 nm wavelength without supplementing with antibiotics at 37°C shaking. We next cultivated 100 µL of log phase culture in fresh 3 mL of BHI at 43°C, 37°C, and room temperature (approximately 25°C) shaking. These were cultivated for at least seven hours and the OD was recorded every hour. We added 0.1 mM IPTG to overnight and log phase cultures for experiments assessing strains using the expression plasmid.

Salt stress assay

Overnight cultures were grown in BHI at 37°C shaking and supplemented with antibiotics as specified. Then, we grew to log phase at an optical density (OD) of 0.4-0.5 at a 600 nm wavelength at 37°C shaking. Following that, we cultivated 100 µL of log phase culture in fresh 3 mL of BHI with 1%, 2%, 3%, 4%, or 6% NaCl and incubated them at 37°C shaking. These were cultivated for a minimum of seven hours, and the OD recorded every hour. When assessing strains with expression plasmid, we added 0.1 mM IPTG to all of the cultures including the overnight, the log phase, and the experiment tubes.

Minimum inhibitory concentration assay

Overnight cultures were grown in BHI at 37°C shaking and supplemented with antibiotics as specified. Then, we grew to log phase at an optical density (OD) of 0.4-0.5 at a 600 nm wavelength. The log phase culture was diluted to the final concentration of 1:20. The assays were conducted in a 96-well plate that was incubated overnight at 37°C under static conditions for about 16-20 hours. After incubation, the optical density (OD) of each well was measured at a wavelength of 600 nm. We added 0.1 mM IPTG to all the cultures throughout the assay for the experiments evaluating strains containing the expression plasmid.

H_2O_2 assays

Overnight cultures were grown in BHI at 37°C shaking and supplemented with antibiotics as specified. Then, we grew to log phase culture at an optical density (OD) of 0.4-0.5 at a 600 nm wavelength. The log phase culture was diluted to the final concentration of 1:20 in BHI with H_2O_2 at the indicated concentrations. The assays were conducted in a 96-well plate that was incubated overnight at 37°C under static conditions for 16-20 hours. After incubation, the optical density (OD) of each well was measured and recorded at a wavelength of 600 nm.

In vivo Galleria mellonella survival assays

G. mellonella worms were ordered from Rainbow Mealworms and placed at room temperature before the assays. The worms were weighed, and those weighing between 180 and 225 mg were recorded and divided into groups with 8-10 worms per group. Overnight cultures were grown in BHI at 37°C shaking and supplemented with antibiotics as specified. Then, we grew to log phase culture at an optical density (OD) of 0.4-0.5 at a 600 nm wavelength. We took 1mL of log phase culture which was pelleted and removed the supernatant fluid. Then, we washed the pellet with

phosphate buffered saline (PBS) and resuspended in PBS. We repeated the second wash with PBS and then diluted the solution to 1:2 dilution. Each worm was injected with 10μL (approximately 10^7 cfu/ml) of the 1:2 dilution and the number of dead worms were recorded every 24 hours for a total of 72 hours. The corresponding 1:2 dilution was also used to complete a 1:10 serial dilution, and the 1:20, 1:200, and 1:2000 dilutions were plated to determine the approximate cfu of culture injected into the worms. These plates were incubated overnight at 30^{o}C and cfu count was determined.

Results

ClpX mediates acid stress via the ClpXP protease

To investigate how the loss of *clpX* affects susceptibility to an acidic pH, we conducted the acid stress assay on two bacterial strains at either neutral pH (7.25) or acidic pH (4.25) and assessed their survival over time (figure 1A). We found a significant difference in survival between the wild-type *B. anthracis* Sterne and *ΔclpX* mutant at acidic pH.

To further examine whether the above phenotype is mediated through the ClpXP protease or through ClpX- independent mechanisms, we repeated the assay with wild-type *B. anthracis*, *ΔclpX* and *ΔclpX* complemented with either wildtype *clpX* gene or the mutant version of *clpX* with a single acid mutation that prevents ClpX from interacting with ClpP (*clpX*^{I265E}) and assessed their survival over time (figure 1B). Because the *ΔclpX* mutant complemented with wildtype *clpX* gene had a different starting number of bacteria at time zero, we normalized the data by looking at % survival over time (figure 1C). As before, we found a significant difference in survival between the wild-type *B. anthracis* with empty plasmid and *ΔclpX* with empty plasmid. This survival defect was rescued in the mutant complemented with plasmid carrying wild-type *clpX* (figure 1C). Nevertheless, when *ΔclpX* mutant was complemented with plasmid

carrying *clpX*^{I265E}, the survival was the same as *ΔclpX* mutant at acidic pH. As a result, we conclude that ClpX-mediated acidic pH resistance is dependent upon the interaction with ClpP through the ClpXP protease rather than through ClpX- independent mechanisms.

Since the ClpXP protease complex is important for the growth of *B. anthracis* in an acidic environment, we next investigated whether loss of either *clpP1* or *clpP2* exhibits the same effect. To test this, we conducted an acid stress assay on the wild-type *B. anthracis*, *ΔclpX*, *ΔclpP1,* and *ΔclpP2* mutants at acidic pH (4.25) and assessed their survival over time (figure 1D). We found that there was a significant difference in survival between the wild-type *B. anthracis* Sterne and *ΔclpP1* or *ΔclpP2* mutants at an acidic pH (figure 1E). This indicates that both ClpP subunits are important for mediating acid stress along with ClpX.

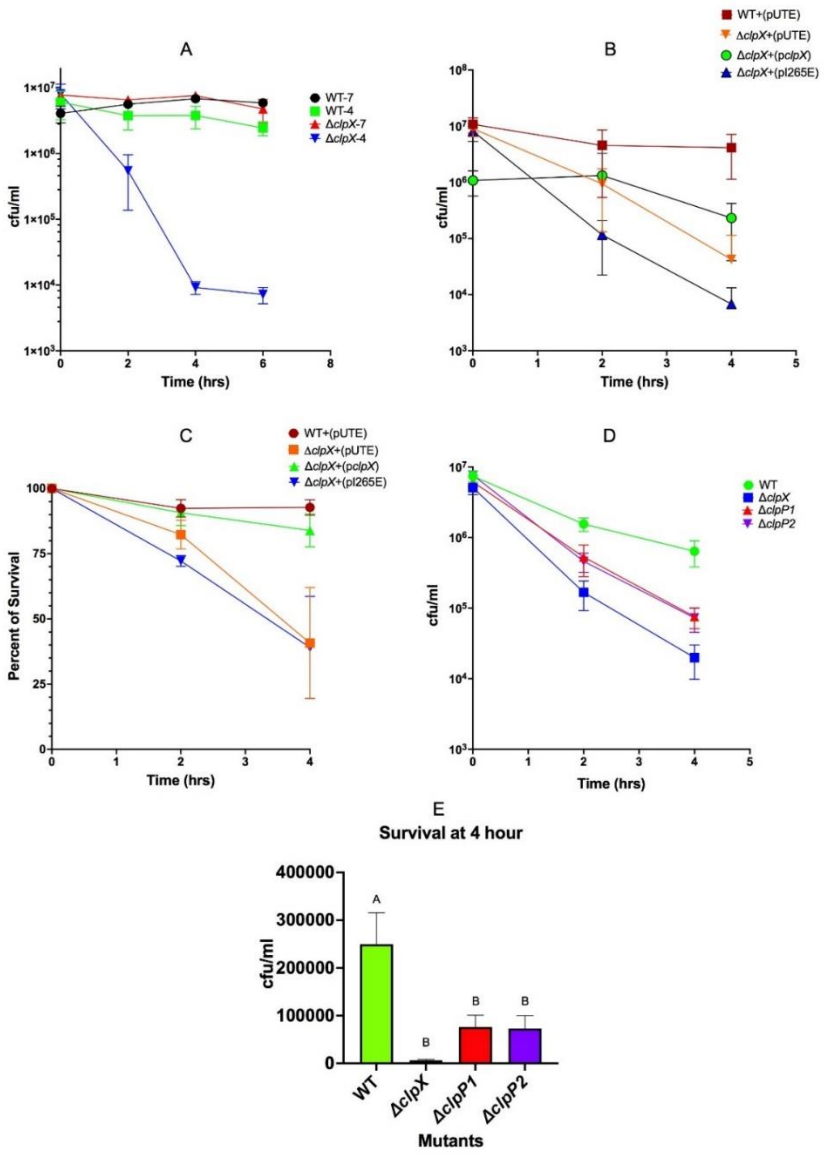


Figure 1: Loss of ClpXP protease through deletion of *clpX*, *clpP1*, or *clpP2* increases the bacterial susceptibility to an acidic environment.

a) Survival assay of the wild-type *B. anthracis* and *ΔclpX* mutants at pH7 or pH4. b) Complementation assay with the wild-type *B. anthracis* carrying inducible plasmid pUTE657 (WT+pUTE), *ΔclpX* mutant containing empty pUTE657 (*ΔclpX* + pUTE), *ΔclpX* containing the wild-type *clpX* gene in pUTE657 (*ΔclpX* + p*clpX*), and *ΔclpX* containing the mutated *clpX* gene in pUTE657 (*ΔclpX* + pI265E) at pH4. c) normalized data from b that measure the percent of survival. d) Survival assay of the wild-type *B. anthracis*, *ΔclpX*, *ΔclpP1*, and *ΔclpP2* mutants at pH4. Data is presented as mean +/- SEM and assays were repeated at least 3 independent times.

*Different letters indicate the means are significant different from each other. One way ANOVA followed by Tukey-Kramer post hoc analysis was used to test whether there is significant difference among these mutants.

ClpX and ClpP are required for growth under heat stress but not cold stress

To evaluate the contribution of *clpX*, *clpP1,* and *clpP2* to temperature stress in *B. anthracis* Sterne, we compared the growth of mutant strains with the wild-type *B. anthracis* under heat and cold stress. Comparison of growth kinetics show a similar growth curve pattern of all the mutants under cold stress (room temperature, approximately 25^{o}C), or under the normal physiological body temperature of 37^{o}C (Figure 2A and 2B). At an elevated temperature of 43^{o}C (heat stress), all the mutants grow slowly in comparison with the wild-type *B. anthracis* (figure 2C). These results suggest that *clpX, clpP1,* and *clpP2* are required for growth during heat stress but not cold stress.

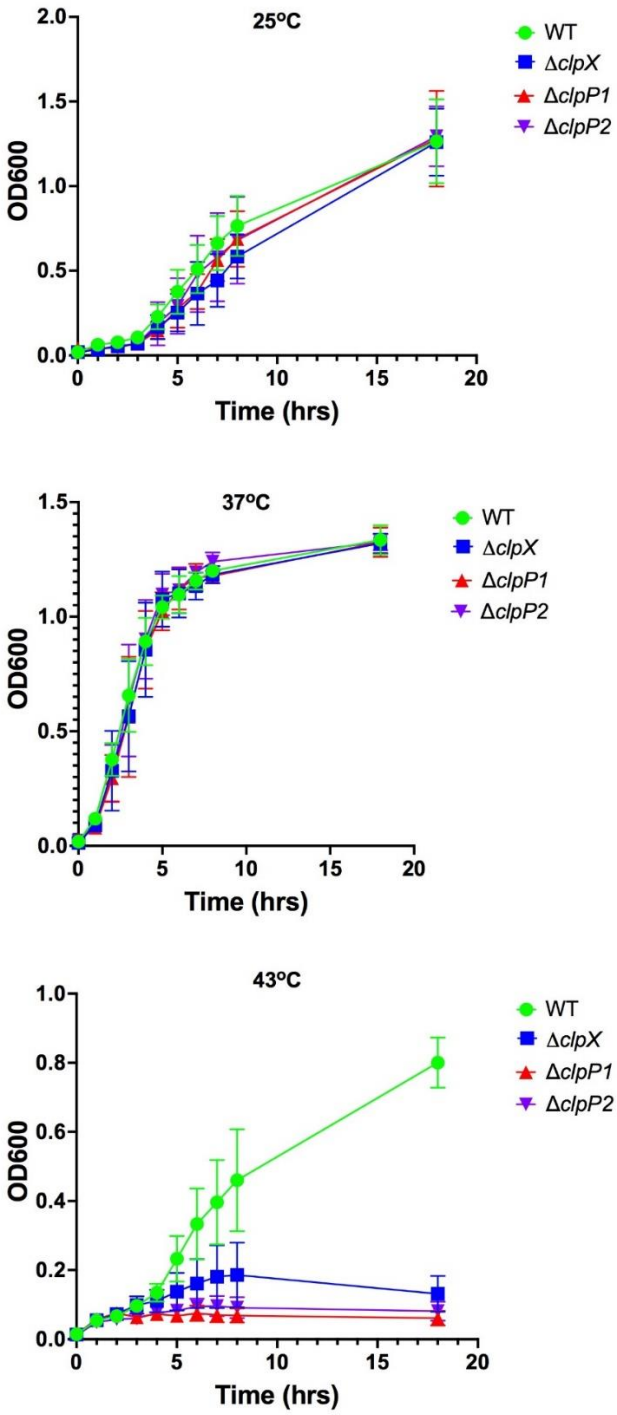


Figure 2: *clpX*, *clpP1*, or *clpP2* are essential for the growth under high temperatures.

Wild-type *B. anthracis*, *ΔclpX*, *ΔclpP1*, and *ΔclpP2* mutants were grown in BHI at a) 25ºC (cold stress) b) 37ºC (physiological temperature), c) 43ºC (heat stress). The absorbance was measured every hour for 8 hours. Data is presented as mean +/- SEM and the experiment was repeated at least 3 times.

clpX is not required for growth under salt stress

Next, we investigated whether *clpX* is required for growth under high concentrations of salt by comparing the growth of *ΔclpX* with the wild-type *B. anthracis* Sterne. To start, we first determined the range of additional NaCl that could be used for the assay. We grew the wild-type *B. anthracis* overnight and then took 100 µL of overnight culture to grow in various concentrations of NaCl (Figure 3A). We found that adding 4% NaCl is the maximum saline concentration before growth was affected. We then compared the growth pattern of wild-type *B. anthracis* Sterne and *ΔclpX* in a range of saline concentrations. We found that both strains had similar growth in the presence of 2% and 4% saline, and no strains survived in the 6% NaCl (figure 3B-D). We conclude that *clpX* is not required for bacterial growth under high salt stress.

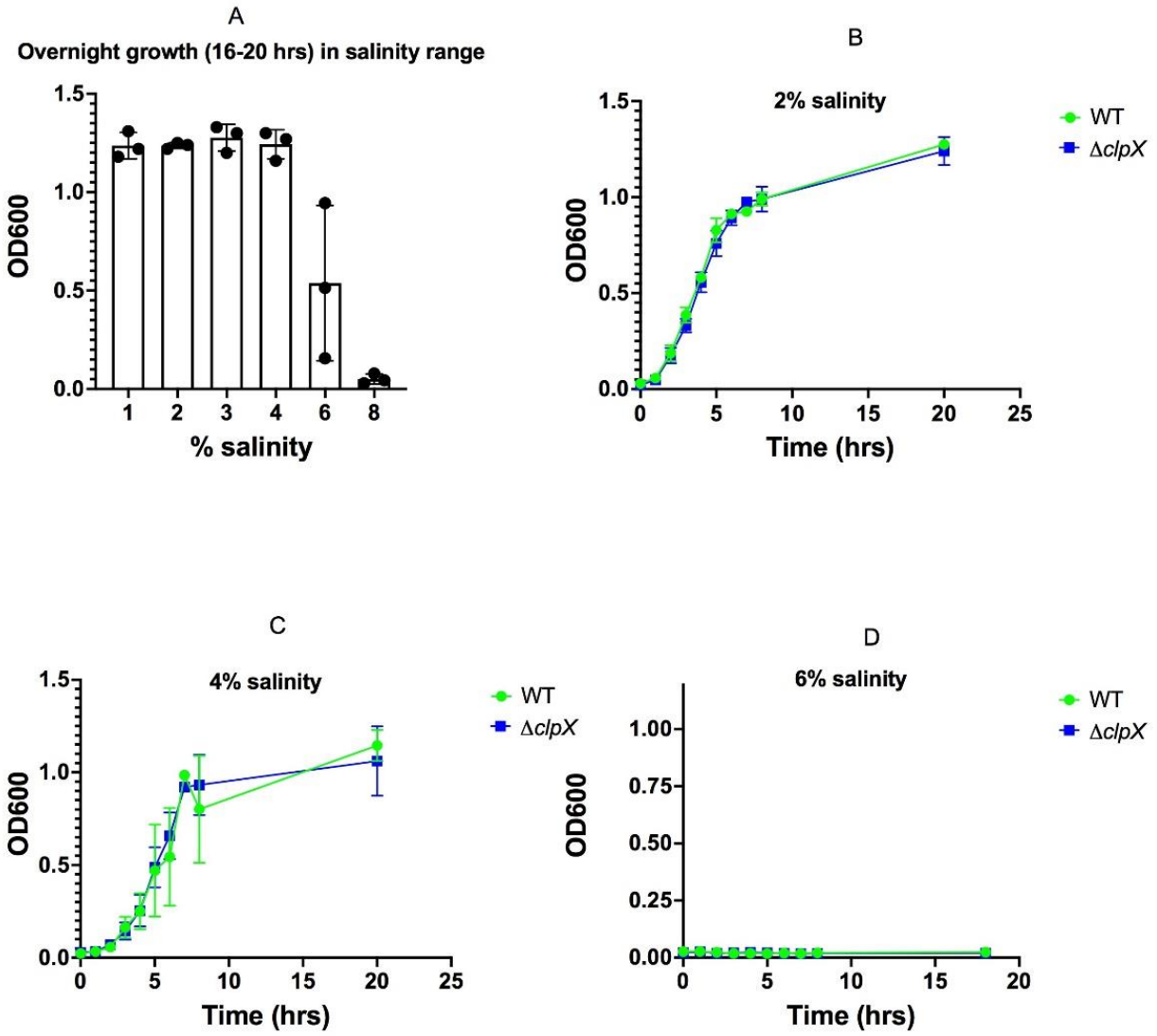


Figure 3: *clpX* does not contribute to the resistance to salt stress.

Wild-type *B. anthracis* and *ΔclpX* mutant were grown in BHI with supplemental NaCl at indicated percent. a) Range of added NaCl that the bacteria could tolerate. b) 2% added NaCl c) 4% added NaCl d) 6% added NaCl. Data is presented as mean +/- SEM and the experiment was repeated at least 3 independent times.

Loss of *clpX* does not affect the resistance to non-cell envelope active antibiotics

As we learned from previous work, *clpX* is required for resistance to antibiotics with cell-envelope targeting activity such as penicillin or vancomycin, as well as antimicrobial peptides such as daptomycin and LL-37 [6, 10]. We were interested in determining if *clpX* is also required for antibiotic resistance to non-cell envelope active antibiotics. Tetracycline and streptomycin inhibit protein synthesis by targeting the ribosome, while ciprofloxacin inhibits DNA synthesis by targeting DNA gyrase. With all non-cell envelope active antibiotics we tested, we found no difference between wild-type *B. anthracis* and the *ΔclpX* mutant (Figure 4A-C). We conclude that ClpX is primarily important for resistance to cell-envelope targeting antibiotics.

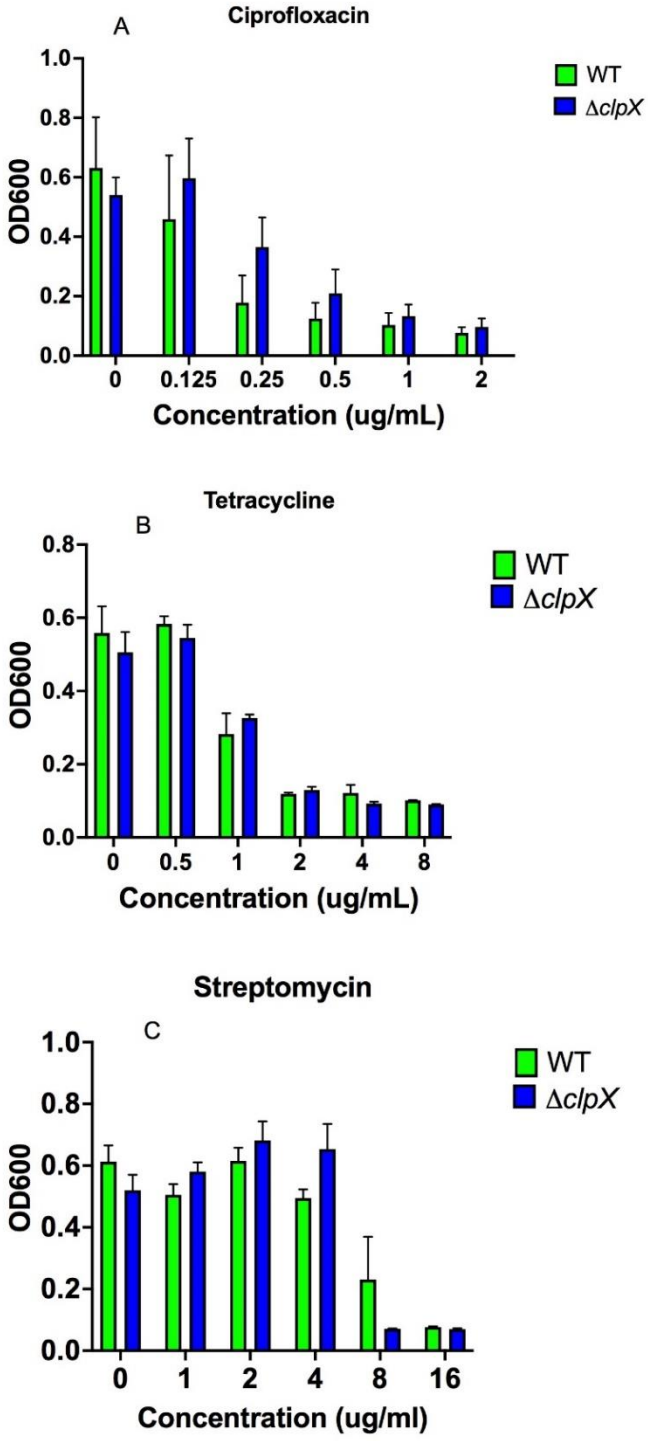


Figure 4: *clpX* does not contribute to the resistance of non-cell envelope active antibiotics.

Growth of wild-type *B. anthracis* Sterne, and *ΔclpX* mutant in media containing either a) ciprofloxacin, b) tetracycline, and c) streptomycin at indicated concentrations. Data is represented as mean +/- SEM, and the experiment was repeated at least 3 independent times. There is no statistical difference between groups determined by student's t-test.

Construction of ClpB and ClpC insertional mutant in *B. anthracis*

We discovered that ClpX plays a critical function in *B. anthracis*; however, the roles of two other members of the Clp-ATPase family, ClpB and ClpC, in *B. anthracis* remain unknown. The second aim of this study is to examine the role of ClpB and ClpC in the control of *B. anthracis* stress responses. To investigate their function, we used insertional mutagenesis to disrupt the *clpB* or *clpC* genes in *B. anthracis* Sterne. We amplified approximately 500 bp of DNA in the middle of the *clpB* gene and sub-cloned it into pHY304, which is a temperature sensitive plasmid. The generated vector was then transformed into wild-type *B. anthracis* Sterne where it integrated into the middle of the *clpB* gene through homologous recombination (figure 5A). PCR was used to verify the insertion and knockout using pHY304 Fwd, a plasmid-specific primer, and a genomic primer downstream of the plasmid insertion site (ClpB IM-2 confirm Rev for clpB). Only when the plasmid was inserted appropriately into the gene can amplification with these primers occur. As seen in Figure 5B, the *ΔclpB* mutant exhibited a band, which was further validated by sequencing. This confirmed the plasmid insertion and *clpB* gene disruption. We also attempted to construct the insertional mutant for *ΔclpC* in a similar manner (figure 5A). Even though the plasmid was successfully transformed into *B. anthracis* Sterne, we were unable to integrate it into the center of the *clpC* gene. As a result, our second objective is to focus only on the *ΔclpB* mutant in the control of the *B. anthracis* stress responses.

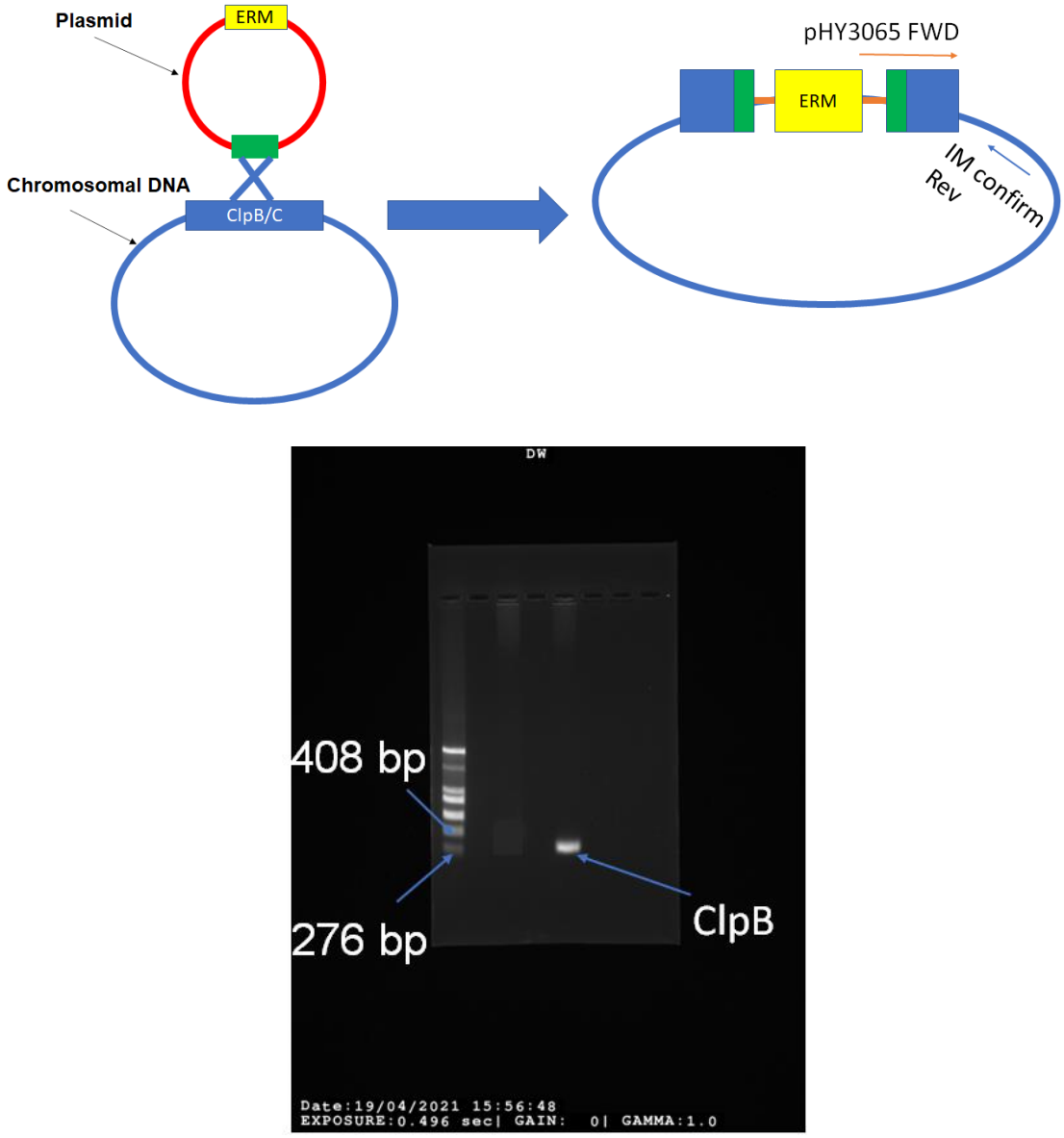
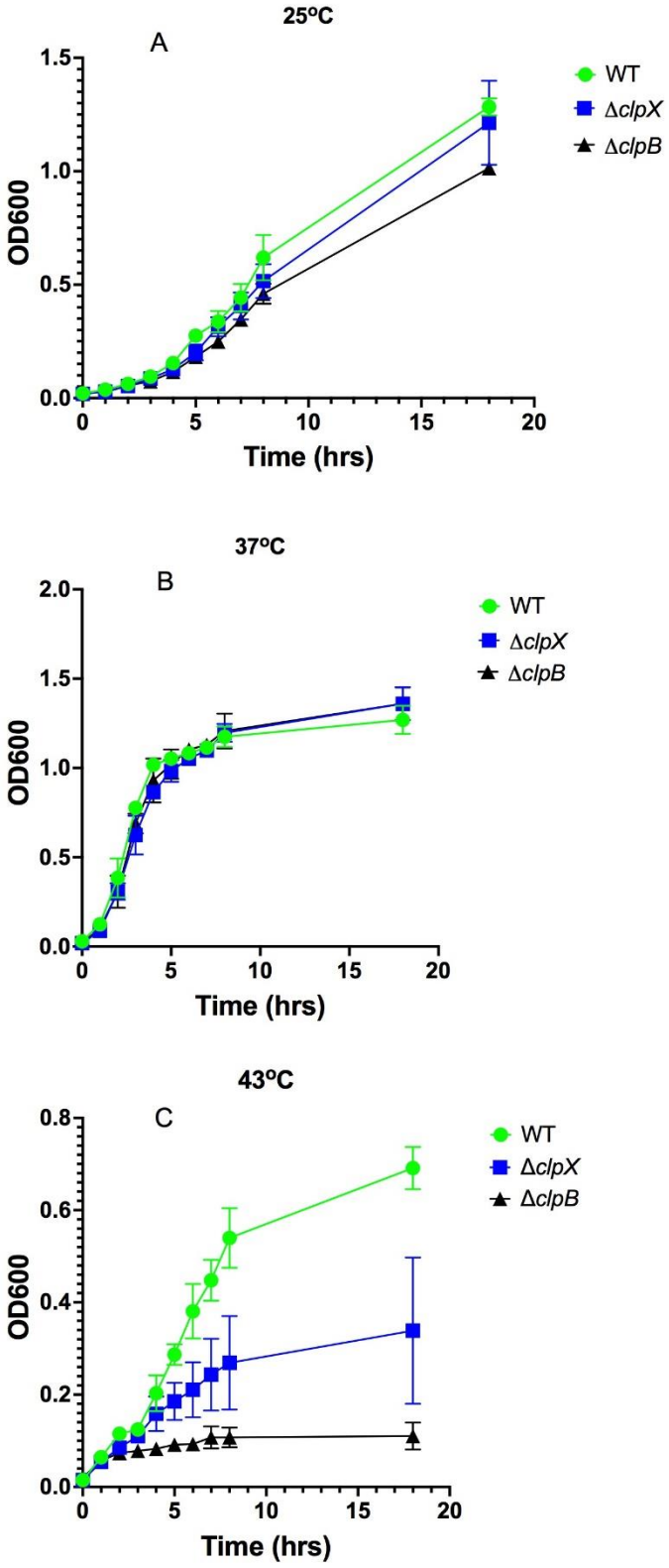


Figure 5: The construction of *clpB/C* insertional mutant.

a) The schematic of the *clpB/clpC* gene are disrupted by integrating a plasmid with the antibiotic resistance gene erythromycin. b) Gel electrophoresis confirmation of plasmid insertion into *B. anthracis* chromosome using pHY3065 Fwd and *B. anthracis* ClpB IM-2 confirm Rev primers.

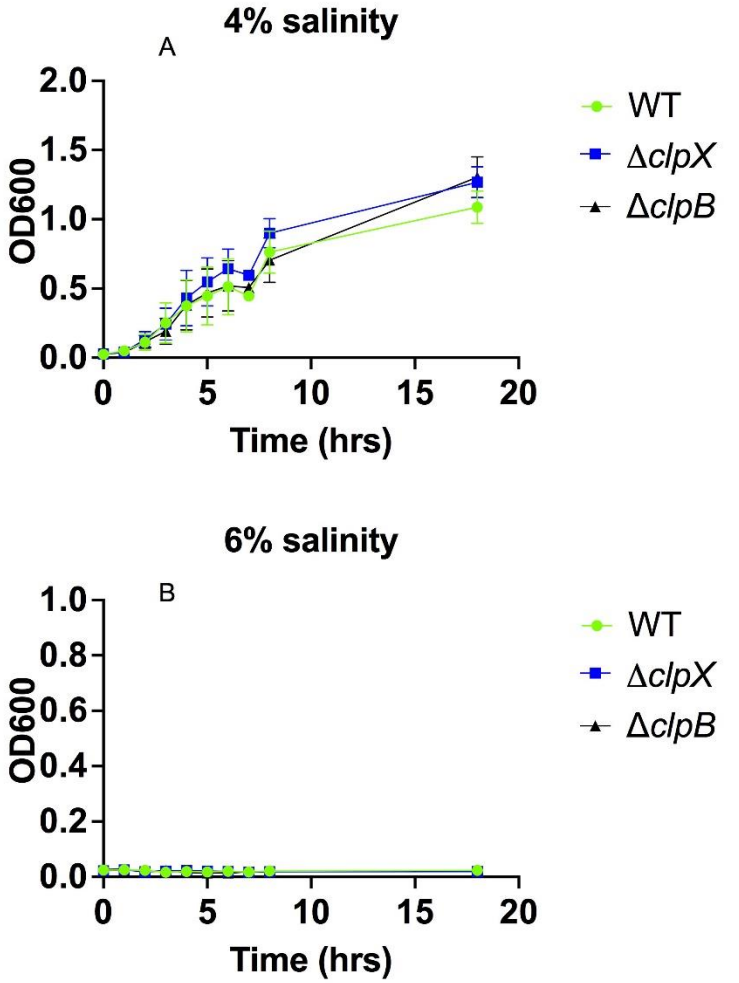


ClpB is required for growth under heat stress but not cold stress, salt stress, or acid stress.

To investigate the effects of *clpB* loss on a variety of stressors, we performed identical stress experiments with two bacterial strains: wild-type *B. anthracis* Sterne and the Δ*clpB* mutant. We observed that ClpB is necessary for the growth of *B. anthracis* Sterne under heat stress conditions, but not when exposed to cold stress (25ºC) or under physiological temperature (37ºC) (Figure 6A-C).

Figure 6: *clpB* is required for the growth in the condition of heat stress.

The wild-type *B. anthracis* Sterne, Δ*clpX*, and Δ*clpB* mutants were grown in BHI at a) 25ºC (cold stress), b) 37ºC (physiological), and c) 43ºC (heat stress). Data is presented as mean +/- SEM and the assay was repeated at least 3 independent times.



We next repeated the salt stress experiment in the same manner as before and we found no change in growth between strains. Therefore, we conclude that there is no relationship between *clpB* and salt tolerance in *B. anthracis* Sterne (figure 7A-B).

Figure 7: *clpB* does not contribute to the resistance of salt stress.

Wild-type *B. anthracis*, *ΔclpX*, and *ΔclpB* mutants were grown in BHI at different % NaCl added. a) 4% added NaCl, and b) 6% added NaCl. Data is presented as mean +/- SEM and the experiment was repeated at least 3 independent times

We then repeated the acid stress experiment in the same manner as before and we found no statistically significant difference in survival between the wild-type *B. anthracis* Sterne and the *ΔclpB* mutant in the acid stress experiment when performed at an acidic pH (figure 8).

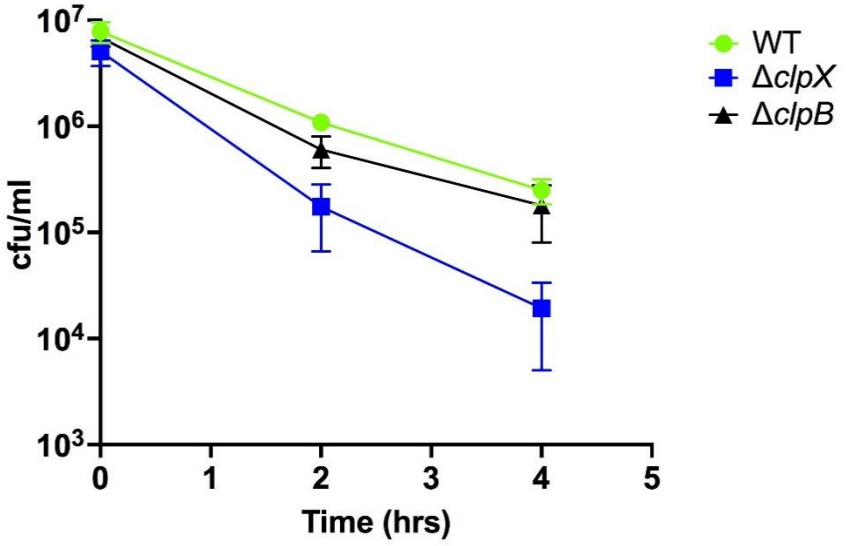


Figure 8: Deletion of *clpB* gene does not affect the survival of *B. anthracis* in acidic environment. Survival assay of the wild-type, *ΔclpB*, and *ΔclpX* mutants at pH4. Data is presented as mean +/- SEM and assays were repeated at least 3 independent times.

ClpB does not contribute to the resistance of either cell-envelope or non-cell envelope targeting antibiotics.

We next tested whether *clpB* plays a role in the control of antibiotic resistance. Our MIC experiments were carried out on the wild-type *B. anthracis* Sterne, *ΔclpX*, and *ΔclpB* strains that had been treated with the cell-envelope active antibiotics penicillin and daptomycin and the non-cell envelope active antibiotics ciprofloxacin, streptomycin, and tetracycline. We discovered no statistically significant difference between wild-type and Δ*clpB* mutant for any antibiotics that we tested (figure 9A-F). We conclude that ClpB does not play a significant role in antibiotic resistance.

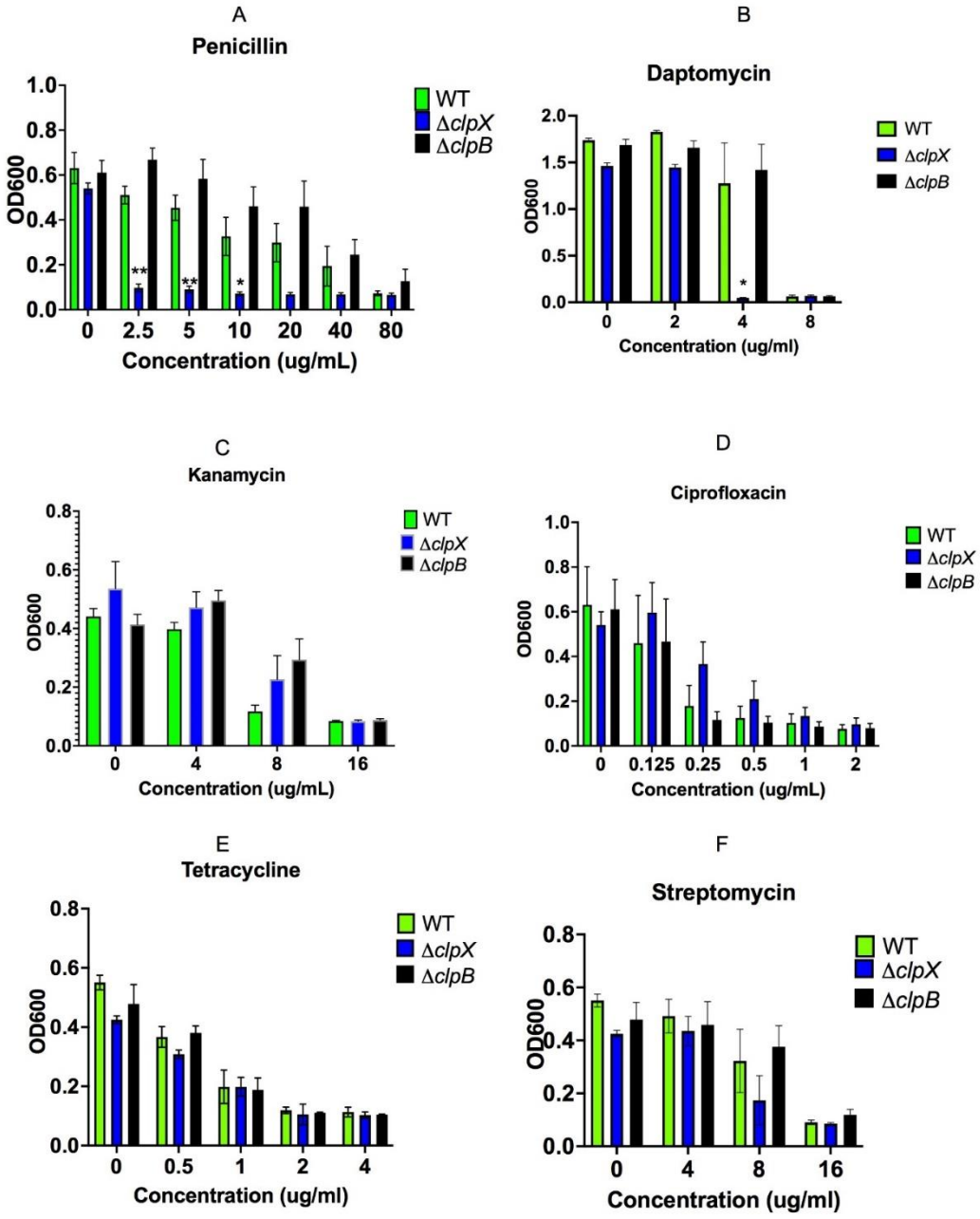


Figure 9: Loss of *clpB* does not contribute to the antimicrobial susceptibility to either cell-envelope or non-cell envelope targeting antibiotics.

Growth of wild-type *B. anthracis* Sterne, *ΔclpX*, and *ΔclpB* mutants in BHI media containing either a) penicillin, b) daptomycin, c) kanamycin, d) ciprofloxacin, e) tetracycline, and f) streptomycin at indicated concentrations. Data is represented as mean +/- SEM, and the experiment was repeated at least 3 independent times.

* Indicate the means are significant different from the wild-type. One-way ANOVA followed by Tukey-Kramer post hoc analysis was used for statistical test.

clpX and *clpB* do not play role in defense against oxidative stress

Next, we wanted to investigate if *clpX* or *clpB* are essential for defense against oxidative stress. To accomplish that, we tested the wild-type *B. anthracis* Sterne, *ΔclpX*, and *ΔclpB* mutants in the presence of H_2O_2 at indicated concentrations. We found that there is no significant difference between the wild-type and any mutants when treated with H_2O_2 (figure 10). We conclude that *clpX* and *clpB* are not crucial for the defense against oxidative stress that was induced by H_2O_2.

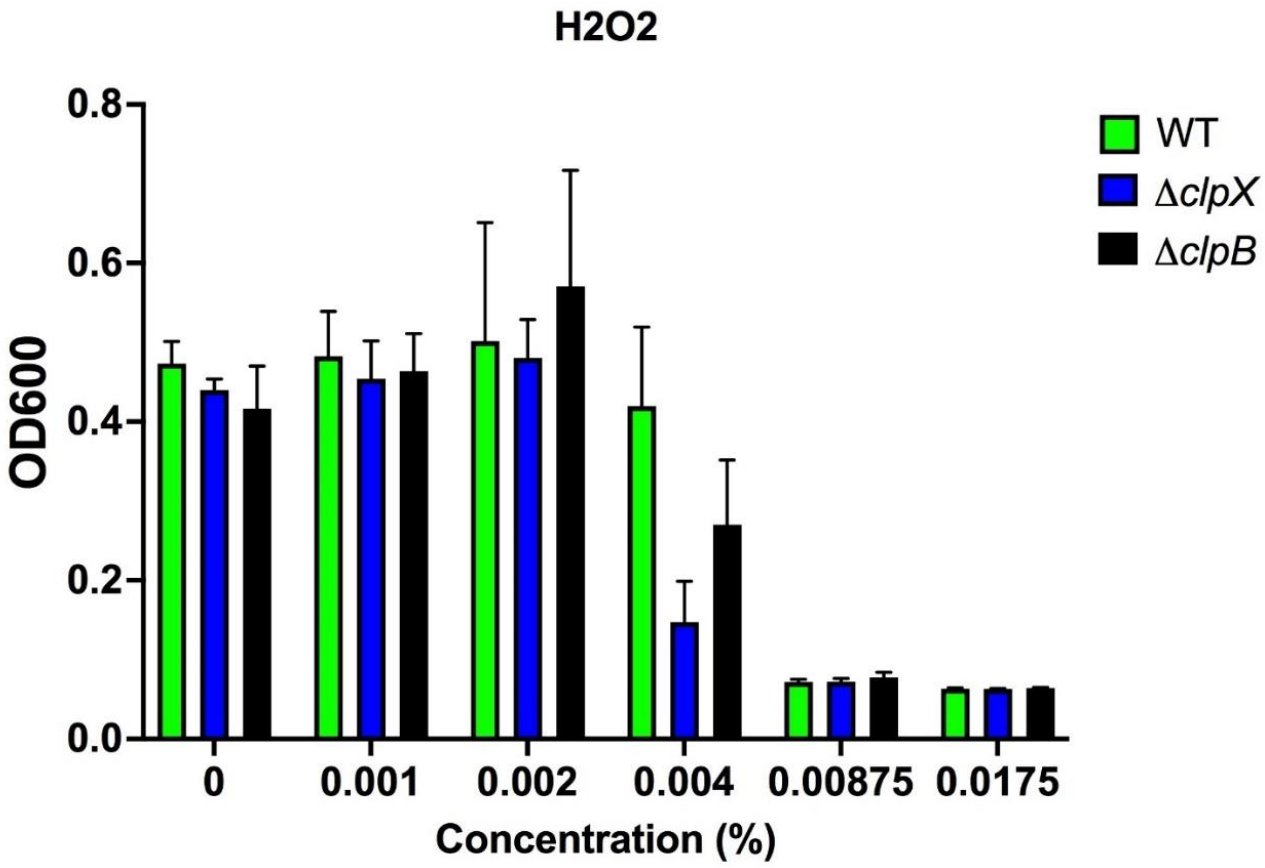


Figure 10: *clpX* and *clpB* does not contribute to the resistance to H_2O_2.

Growth of wild-type *B. anthracis* Sterne, *ΔclpX*, and *ΔclpB* mutants in BHI media containing H_2O_2 at indicated concentrations. Data is presented as mean +/- SEM and the assay was repeated at least 3 independent times. One-way ANOVA followed by Tukey-Kramer post hoc analysis was used to test whether there are any statical significant difference among groups.

clpB does not contribute to the virulence of *B. anthracis* in *G. mellonella*

Lastly, to assess the consequences of interrupting *clpB* in animal survival, we chose the waxworm *G. mellonella,* which is regarded a viable *in vivo* model for *B. anthracis* infection research since it has parallels to the human innate immune system [39]. We split the larval form of *G. mellonella* into treatments group of eight to ten weighing 180-225 mg each to minimize the effects produced by variation in weight. The bacterial overnight cultures were grown until log phase (OD ~ 0.4-0.5), washed and diluted 1:2 in PBS for injection. The quantity of dead larva was simple to discern since the dead larva became black after dying (figure 11A), and larval survival was examined every 24 hours for a total of 72 hours. As predicted, the larval group injected with PBS exhibited a high survival rate whereas the group injected with wild-type *B. anthracis* Sterne had the greatest death rate (figure 11B). The group with the Δ*clpX* mutant injection had a modest survival rate and is significantly different from the wild-type group (figure 11B). We found that there is no statistically significant variation in survival rate between the group receiving the Δ*clpB* mutant injection and the wild-type. We conclude that deletion of Δ*clpB* does not have an effect on virulence of *B. anthracis*.

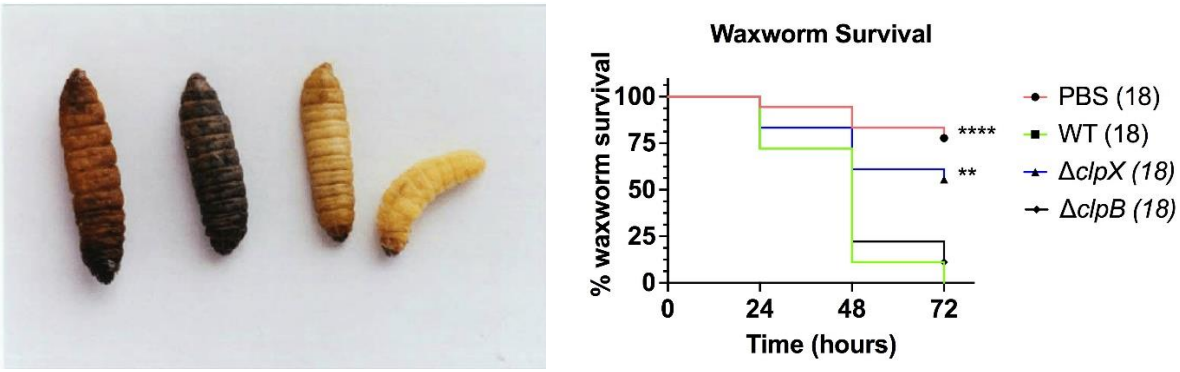


Figure 11: Loss of *clpB* does not show attenuated virulence of *B. anthracis* in *G. mellonella*

a) The illustration depicts infected *G. mellonella* with dead larvae (dark-colored) and live larvae (yellowish). b) Survival rate of waxworms injected with PBS, wild-type *B. anthracis* Sterne (WT), Δ*clpX* and Δ*clpB* mutants at 24, 48, and 72 hours. The experiments were conducted twice independently with eight to ten larvae for each condition, and the total number of worms for each condition is shown between parentheses.

** indicates $p<0.01$, **** indicates $p<0.0001$ from the wild-type *B. anthracis* Sterne survival using the log-rank test.

Discussion

Upon infection of a host, bacterial pathogens experience major changes in their environment relating to pH, temperature, and osmolarity, which may ultimately lead to a conformational change in several essential bacterial proteins, resulting in proteins that are misfolded. It may result in nonfunctional proteins, random aggregation, and ultimately cell death. To provide cell protection during stress reactions, bacterial cells express chaperone proteins that preserve normal protein folding or degrade unfolded proteins [40]. Moreover, host inflammatory reactions engage phagocytic cells, which expose pathogens to detrimental conditions, such as oxidative stress and antimicrobial peptides. Consequently, bacterial survival relies on molecular adaptations, also known as stress responses, to deal with these harsh environments and bacterial chaperone systems play crucial roles in this regard.

Previous investigations showed that ClpX, an ATPase that acts as a chaperone and partner to the ClpP protease, is essential for the virulence of *B. anthracis* and resistance against

antimicrobial peptides and cell-wall active antibiotics [6, 10]. We hypothesized that ClpX may also be essential for the regulation of other stress responses, including acid stress, temperature stress, salt stress, oxidative stress, and non-cell envelope active antibiotic stress in part because ClpX plays an important role in regulation of these stress responses in other pathogens [19, 20]. We found that ClpX plays a role in acid and heat stress, but not cold stress, salt stress, oxidative stress or non-cell envelope active antibiotics. We then looked whether the phenotype is mediated through protease activity or chaperone activity of ClpX. We found that *ΔclpX* complemented with the wildtype *clpX* gene recovers the ability to survive in acidic environment, whereas complementing with the mutant version $clpX^{I265E}$ does not (figure 1B-C). This demonstrated that ClpX interacts with ClpP as a protease to facilitate *B. anthracis* survival in an acidic environment. We also found that either *clpP1* or *clpP2* are required for *B. anthracis* growth under heat stress but not cold stress (figure 2), which indicates that protease activity also contributes to mediating this stress response, although we have not yet tested whether ClpX chaperone could also play a role. This will be tested using *ΔclpX* complemented with the wildtype *clpX* gene or complemented with the mutated *clpX* gene. It is notable that for all the ClpX-mediated stress responses we have evaluated ClpX and ClpP work in tandem. As a result, the protease mechanism of ClpX seems to be the most important in terms of regulation of stress responses in *B. anthracis*. This is slightly different from *S. aureus* in which there are ClpX associated phenotypes that are mediated through chaperone activity such as decreased susceptibility of β-lactam antibiotics [41]. However, the majority of cell physiology in *S. aureus* is mediated through the action of ClpXP protease including the attenuation in virulence so this is likely the major mechanism [42].

We also discovered that deletion of the individual *clpP* subunits, which also causes ClpXP protease malfunction, contributes to a similar phenotype as deletion of *clpX* (figure 1D).

The deletion of *clpX* seems to be more detrimental than loss of either *clpP1* or *clpP2*, although there is no significant difference between *ΔclpX* mutant and both *clpP1* or *clpP2* in acid stress as there is a striking trend in decreased recovery with *ΔclpX* relative to *ΔclpP1 or ΔclpP2*, although the difference is not statistically significant (figure 1E). This is very similar to what we discovered in antibiotic resistance, where loss of either *clpP1* or *clpP2* contributes to cell-envelope antibiotic sensitivity, but not to the same extent as loss of *clpX* [39]. This suggests that ClpP1 and ClpP2 may partly compensate for each other, and that double knockout of both *clpP* subunits is likely needed to have the same impact as loss of *clpX*. This result is crucial because ClpP subunits might be exploited as an efficient therapeutic target for combating anthrax infection; however, both subunits would likely need to be targeted for the therapeutic benefits to be maximized. In *M. tuberculosis*, *clpP1* and *clpP2* are co-transcribed in a single operon and operate in tandem to degrade proteins [16]. When ClpATPase interacts with a single ClpP1 or ClpP2 protease, it may attach to either side of the protease [43]. Nevertheless, ClpP1 and ClpP2 may form a heteromeric tetradecamer ring that can also interact with ClpATPase, therefore promoting ATPase activity, peptide cleavage, and protein degradation [43]. In addition, ClpX and ClpC preferentially bind to the ClpP2 side of the complex in *M. tuberculosis* [44].

After clarifying the role of *clpX* in stress responses, we wanted to determine the function of the other ClpATPase family members, *clpB* and *clpC*, in *B. anthracis* as well. We found that *clpB* is essential for *B. anthracis* to grow under heat stress (figure 6); however, it does not play any role in regulation of other stress responses including cold stress, acid stress, salt stress, cell-envelope/non-cell envelope antibiotic resistance, or oxidative stress nor does it play a role in virulence in our *G. mellonella* infection model (figure 7-11). A competition model of virulence, where both the wild-type and *ΔclpB* mutant are injected into a single worm for direct growth competition, is a more sensitive assay than the survival model we used in this study [39]. It is

possible that a competition virulence assay between WT and Δ*clpB* may discern a virulence defect in the *ΔclpB* mutant, but it is unlikely that ClpB would have the same impact as ClpX on virulence. Overall, *clpB* is necessary for thermotolerance in *B. anthracis*, which is also seen in *S. aureus* and *L. monocytogenes*, however unlike *B. anthracis*, *clpB* is also required for the salt stress in *L. monocytogenes*. In the future, we want to complement the *ΔclpB* mutant with an expression plasmid harboring the wild-type *clpB* gene in order to re-evaluate the phenotype seen under heat stress. This finding of *clpB* in *B. anthracis* might not be helpful to therapeutic perspectives; however, it can be important related to how *B. anthracis* in the spore form can be highly resistant to many adverse conditions including heat stress.

Lastly, we also attempted to construct the insertional mutant *ΔclpC* to identify its role in stress responses. During the process, we successfully transformed *B. anthracis* Sterne with the construct; however, we were unable to integrate the plasmid into the middle of the *clpC* gene to induce the insertional knock-out. The failure to construct the insertional mutant *ΔclpC* could be due to several reasons. It could be our amplified sequence in the plasmid did not integrate into the *clpC* gene but rather into another region of the genome, despite the homology between our targeting plasmid and the *clpC* gene. It is unlikely that the clpC gene is essential for *B. anthracis* though because another research group used the *Cre-loxP* genetic modification method and successfully constructed a knock-out *ΔclpC* mutant. This is a technique that we could try in our lab for the future. We can also try to design a different insertion site for a redesigned insertional mutant, or we may perform a true genetic deletion of *clpC*, which requires two recombination events and a redesign of the targeting plasmid. In *B. anthracis, clpC* is required for growth during heat stress (43^{o}C) and ClpC has been demonstrated to play an essential role in viable spore formation and germination specifically at engulfment stage [22]. It is still unclear the role of *clpC* gene in *B. anthracis* in regulation of stress responses including acid stress, salt stress,

antibiotic resistance, oxidative stress, or virulence. Since ClpC is similar to ClpX in terms of its ability to form a protease complex with ClpP [45], ClpC would be more likely to have a significant phenotype then ClpB and it is worth continuing to investigate the role of this ATPase. Studying the Clp systems could provide us the understanding of the mechanisms and factors contributing pathogenesis and virulence in *B. anthracis* and we may use this knowledge onto other gram-positive bacteria. Gaining a better understanding of the role of Clp ATPase family members in regulation of multiple stress responses will ultimately provide us more targets to either directly fight infection or to help make current antibiotics more effective.

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ABSTRACT

ROLE OF THE CLPATPASE FAMILY MEMBERS IN THE STRESS RESPONSE OF *BACILLUS ANTHRACIS*

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Anthrax is an infectious disease caused by *Bacillus anthracis*, which is a spore forming bacterium. Even though the anthrax toxins and capsule, encoded on 2 plasmids pXO1 and pXO2, play crucial role in the pathogenesis of anthrax infection, evidence suggests that chromosomal genes also play a role. The ClpX ATPase was discovered to be crucial for *B. anthracis* virulence via protection against host antimicrobial peptides. In this study, we want to investigate the role of ClpATPase family members in regulation of stressors including acidic stress, temperature stress, salt stress, and non-cell/cell envelope active antibiotics. We found that *clpX* is necessary for survival in an acidic environment and growth under heat stress. We demonstrate that acidic stress resistance is mediated by the formation of the ClpXP protease using a ClpX complementation plasmid that is incapable of interacting with ClpP. There is no association between *clpX* with other stressors. Additionally, we genetically disrupted other Clp ATPase in *B. anthracis*, ClpB and ClpC, to study its role in the regulation of stress responses. Unfortunately, we was not successful in constructing the insertional knock-out ClpC to study its role. We discovered that *clpB* is necessary for growth under heat stress. Acidic stress, salt stress, antibiotics have no association with *clpB*. We conclude that the ClpX ATPase is required for *B. anthracis*

pathogenicity via defenses against host antimicrobial peptides and for survival in an acidic environment. Understanding the role of members of the Clp ATPase family in the regulation of stress responses will ultimately infer us with more targets for either directly combating infection or improving the efficacy of already available medicines.