INVESTIGATING THE CONNECTION BETWEEN THE CLPXP PROTEASE AND FTSZ IN ANTIMICROBIAL PEPTIDE RESISTANCE IN BACILLUS ANTHRACIS

By

Diem Q. Ngo

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Project Approved:

Supervising Professor: Shauna M. McGillivray, PhD.

Department of Biology

Giridhar R. Akkaraju, PhD.

Department of Biology

Catherine A. Coleman, PhD.

Department of Strategic Communication

ABSTRACT

Anthrax is caused by *Bacillus anthracis*, a deadly gram-positive bacterium that could be used as a potential bioterrorist weapon. *B. anthracis* has virulence factors that contribute to its deadly nature by helping the bacterium evade destruction by the host immune system. One of these factors is a protease subunit called ClpX which was shown to be essential for virulence in animal models. Loss of ClpX also resulted in decreased resistance to antimicrobial peptides, an essential part of innate immunity. However, it is not yet understood why the loss of ClpX results in a loss of resistance to antimicrobial peptides in *B. anthracis.* More recently, we have seen that *B. anthracis* lacking ClpX has aberrant cell division resulting in mini-cell formation. Mini-cell formation can occur when there is too much expression of cell division proteins such as FtsZ. Interestingly, ClpX regulates FtsZ expression in bacterial species such as *E. coli.* For these reasons, we hypothesized that loss of ClpX would lead to overexpression of FtsZ and to mini-cell formation. These mini-cells may be contributing to decreased resistance to antimicrobial peptides. To test this hypothesis, we overexpressed FtsZ using an inducible vector in *B. anthracis* and exposed the cells to increasing concentrations of an antimicrobial peptide called LL-37. However, we found that after exposure to antimicrobial peptides with increased levels of FtsZ *B. anthracis* did not exhibit the same phenotype as the ClpX mutant. Thus, our results suggest that the rise in FtsZ levels may not be a key factor in the loss in *B. anthracis* resistance to antimicrobial peptides in the ClpX mutant.

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TABLE OF CONTENTS

INTRODUCTION

Bacillus anthracis is a gram-positive bacterium that causes the deadly disease anthrax. Anthrax infection is usually transmitted through contact with infected animals and can cause illness that range from flu-like symptoms to death. *B. anthracis* can utilize three different routes to infect humans: gastrointestinal, inhalational, and cutaneous. These different routes can cause a range of different symptoms. Cutaneous transmission usually occurs when the bacterium enters through a break in the skin and may cause swelling, blisters, or sores. *B. anthracis* can also cause gastrointestinal infection when contaminated meat is consumed. Though rare, this form can cause nausea, bloody vomiting, and diarrhea. Lastly, the most fatal form is inhalation of anthrax spores. Inhalation anthrax causes septicemia, vomiting, and even death [1]. The bacteria can travel to the lymph node once inside the body and transition to a more active form and release toxins into the body [1]. Thus, *B. anthracis* spore-forming abilities and potential fatal outcomes make anthrax a concern for bioterrorism. In 2001, five out of twenty-two exposed victims died after coming into contact with anthrax spores intentionally put into the U.S. mail illustrating the deadly nature of this disease

B. anthracis has the ability to cause disease within the human body due to virulence factors produced by the bacterium. Two of the most well-studied virulence factors are the toxins and capsule, encoded on two extra-chromosomal plasmids called pX01 and pX02 respectively. The toxins, lethal toxin and edema toxin, enter the cell by endocytosis. Both toxins disrupt signaling pathways within the cell leading to suppression of the host immune response and cell death [2]. The capsule is another virulence factor that prevents the immune cells from phagocytosing the bacteria, allowing the bacteria to avoid clearance by the immune system [3].

In this study, the Sterne strain of *B. anthracis* was used. This strain does not contain the pX02 plasmid, which carries the capsule virulence factor, and thus is avirulent.

Although these plasmid virulence factors are important for virulence, they are not solely responsible for it. In one study, researchers found evidence that the toxins were not necessary for escaping macrophages once endocytosed [4] which suggested that there may additional factors, including the chromosomal factors, contributing to virulence of *B. anthracis*. One of these chromosomal factors is a global protease subunit called ClpX [5]. ClpX works in conjunction with ClpP to form the ClpXP protease complex which breaks down many different proteins found in *B. anthracis*. The ClpP subunit degrades proteins while ClpX functions as a regulatory ATPase, which recognizes proteins marked for degradation [6]. Loss of ClpX through genetic deletion (ΔClpX) rendered *B. anthracis* avirulent [5]. Not only is ClpXP important for virulence in *B. anthracis*, the ClpXP protease is conserved amongst many bacterial species and has been found to be necessary for virulence in other species such as *Staphylococcus aureus* [6].

While ClpXP is critical for virulence in *B. anthracis*, less is known about its mechanism of action. It is not yet understood what bacterial functions are specifically affected by loss of ClpX. However, current evidence points to alterations in the cell wall. First, the loss of ClpX results in increased susceptibility to antimicrobial peptides (AMPs) like LL-37. [5] Antimicrobial peptides are part of the host innate immune response, which is a first line of defense against pathogens. AMPs work by forming a pore in the cell membrane after passing through the cell wall and thus can lead to detrimental results such as lysis or depolarization of the bacterial cell membrane. While the primary function of AMPs is at the cell membrane, bacteria have developed resistance mechanisms against AMPs that include modulating cell wall charge [7]. The ΔClpX strain also had decreased resistance to other cell wall acting antibiotics such as

penicillin and daptomyocin [8]. The commonality between AMPs and these antibiotics is that they specifically target or interact with the cell well. Therefore, the increased susceptibility seen in ΔClpX strain suggests it may have lost the regulation of its cell wall integrity.

These recent discoveries strongly imply that the ΔClpX strain suffered from changes in the cell wall leading to increased susceptibility of antimicrobials that target the cell wall. In order to visualize the cell wall and any changes that has occurred, the ΔClpX strain was observed using transmission electron microscopy. The ΔClpX mutants had thinner cell walls as well as increased division sites resulting in irregular smaller cells called mini-cells. [9]. The mini-cell formations provide a potential explanation for why ΔClpX loses resistances to cell wall acting agents like LL-37 and daptomycin. In recent years, characterization of the mode of action of these antimicrobials suggests they target the cell envelope by specifically impairing cellular division. For example, LL-37 interferes with synthesis of the cell wall [10]. Nisin, another cell envelope acting antibiotic, also targets the cell wall biogenesis and results in altered morphology near the division site [11]. Similarly, daptomycin recruits cellular division proteins and also causes altered bacterial cell wall morphology [12]. Therefore, these additional division sites seen in ΔClpX could be increasing the targets for the cell wall acting agents subsequently leading to increase susceptibility to these agents.

The exact role the ClpXP protease plays in regulating cell division is not well understand, although ClpXP is known to regulate many proteins including proteins involved in cell division, such as FtsZ [13]. FtsZ participates in cellular division by gathering and forming a ring underneath the cellular membrane, controlling the formation of the septum and ultimately the site of division [14]. Since the ClpXP protease normally degrades FtsZ [13], ClpXP may control the rate of cellular division by controlling the degradation of FtsZ. For example, ClpXP may degrade

FtsZ in order to inhibit cellular division or decrease degradation of FtsZ in order to stimulate cellular division. Therefore, the defects seen in the ΔClpX strain may be due to loss of control of FtsZ leading to increased levels of FtsZ. Furthermore, increased levels of FtsZ in *E. coli* have been seen to induce mini-cell formation [15]. If that is also true in *B. anthracis*, then increased FtsZ levels in *B. anthracis* may be the cause of the mini-cell formation seen in the ΔClpX mutant.

The objective of this study was to understand the role of mini-cell formation in antimicrobial peptide resistance in *B. anthracis*. We hypothesized that increased FtsZ levels would induce the same mini-cells seen in the ΔClpX strain. We tested this hypothesis by overexpressing FtsZ levels using an inducible vector called pUTE-657. Expression of pUTE-657 is controlled by the inducer IPTG. The FtsZ gene was inserted into the pUTE plasmid creating a new plasmid called pFtsZ. By adding IPTG to the pFtsZ plasmid, we increased expression levels of FtsZ in *B. anthracis* in order to test whether overexpression of FtsZ resulted in mini-cells formation and showed loss in resistance to AMPs.

MATERIALS AND METHODS

Bacterial strains and media

The wildtype *B. anthracis* used was the Sterne (pX01+, pX02--) strain and the *B. anthracis* ΔClpX mutant was obtained from previous studies [5]. The parental *B. anthracis* and the ΔClpX cultures were grown in brain heart infusion (BHI) or Roswell Park Memorial Institute medium (RPMI) with 5% lysogeny broth. They were incubated at 37^{o}C shaking unless otherwise stated.

Creation of pFtsZ plasmid and transformation of plasmids into competent *E. coli*

The FtsZ gene was amplified by PCR from the *B. anthracis* genome using the pFtsZ Fwd and Rev primers (listed below) and FtsZ and pUTE-657 were digested with restriction enzymes SphI and SalI (New England BioLabs) followed by ligation using a 5:1 molar ratio of insert to vector. The ligation mixture was then transformed into competent NEB 5-alpha *E. coli* cells using the High Efficiency Transformation Protocol from New England BioLabs. The presence of the plasmid with the FtsZ gene insert, called pFtsZ, was confirmed with PCR using the plasmid specific primers pUTE FWD and pUTE REV. The pFtsZ plasmid was extracted from NEB 5-alpha *E. coli* using the Mini-Prep protocol (Promega). Then pFtsZ and empty pUTE657 were transformed into GM-2163 *E. coli*, which are a methylation deficient strain of *E. coli*. This prevents methylation of the plasmids and will prevent degradation of the plasmids in the *B. anthracis* Sterne strain which degrades methylated DNA. A successful transformation was confirmed with PCR using pUTE FWD and REV primers.

Name of Primers	Sequence
pUTE FWD	GAA CGT TGC TCG AGG GTA AAT G

pUTE REV	GGC TGA AAG ATC GTA CGT ACC
pFtsZ FWD (SalI)	ATGCGTCGACACGTGTATTCCGTTATTTATGGGATTA
pFtsZ REV (SphI)	TGCAGCATGCGCAAAGAGCGGGATAGATTGAA

Transformation of *B. anthracis* Sterne

Overnight cultures of WT and ΔClpX *B. anthracis* were diluted with BHI and grown to an optical density of 0.6 to 0.8 at 600nm. Cells were washed and collected by sterile filtration and then mixed with 1 μg of the pUTE657 or pFtsZ plasmid. Afterwards, the cells were electroporated at 1.8 to 2.0 kV and quickly mixed with recovery media for 1 hour. The solution was then plated on BHI with 100 μg/ml spectinomycin (SPEC) antibiotic and placed at 37^{o}C. Colonies that grew on the BHI SPEC plates were checked for the pUTE and pFtsZ plasmid using the pUTE FWD and REV primers and followed by gel electrophoresis to determine the length of the bands.

RNA extraction, cDNA synthesis, and Quantitative real-time PCR

The RNeasy Mini Kit (Qiagen) was used to extract *B. anthracis* RNA. The procedures of the manufacturer were followed using overnight (stationary phase) cultures in BHI and varying levels of IPTG. The extracted RNA was then treated to two rounds of DNase from the TURBO DNA-free Kit. Afterwards, equal amounts of cDNA were synthesized from the RNA with High Efficiency cDNA Synthesis Kit (Applied Biosystems) using the manufacturer's protocol. QPCR was conducted using SYBR Green PCR Mix (Life Technologies). FWD and REV short primers

for the appropriate genes (FtsZ and GryB) were used. GyrB gene was used to normalized expression of gene expression in *B. anthracis*.

Conditions:

Stage	Repetitions	Temperature	Time
1	1	50^{o}C	2:00
2	1	95^{o}C	10:00
3	40	95^{o}C	0:15
		60^{o}C	1:00

Primers used are listed in the table below:

Name of Primers	Sequence
FtsZ FWD	CGT GCA ACA CAA GCG GCA TC
FtsZ REV short	CGC CAA TGT TAG AGG CAT TCC GTG
GyrB FWD	GGT GTT GGG GCA TCT GTA GT
GyrB REV	GTA TTC CGG TTG CGG ATT TA

The Livak method was used to calculate the relative expression of genes (Bio-Rad Laboratories, *Relative Quantification Normalized Against a Reference Gene* 2012). The calculations are briefly described below.

$$\Delta\Delta CT = \Delta CT_{(\text{Target Gene})} - CT_{(\text{reference gene})}$$

$$\text{Normalized Expression} = 2^{-\Delta\Delta CT}$$

Growth assays

All the strains were grown in the presence of 1mM of IPTG during the stationary phase, log phase, and in the growth assay. Bacterial cultures taken from stationary phase were grown to log phase (optical density of 0.4 at 600 nM) in BHI. The log-phase cells were pelleted by centrifugation and resuspended in 1X PBS. Then the PBS bacterial cultures were diluted 1:20 in RPMI. The RPMI dilutions were then plated into individual wells on a 98 well assay plate. Antibiotics were added for a final concentration of 0 μM, 0.8 μM and 1.6 μM of LL-37 antimicrobial peptides with a total volume of 200 μL in each well. The assay was placed into the 37^{o}C incubator for 24 hours. Reading of the assay occurred at 24 and 48 hours after completion of the assay.

Gram-Staining Bacterial Strains

A smear from a bacterial colony was diluted in several drops of water and heat fixed onto a microscope slide. Then the slides were stained with crystal violet and iodine, and then washed with water and decolorizer. The slides were then viewed under a light microscope using oil immersion at 1000x magnification.

RESULTS

Confirmation of transformation into B. anthracis

The plasmid pUTE-657 was transformed into both WT and ΔClpX *B. anthracis* strains and the pFtsZ plasmid was transformed into WT *B. anthracis.* These plasmids are induced by IPTG but only the WT pFtsZ strains have the FtsZ gene inserted. Therefore, only the pFtsZ strain can overexpress the FtsZ protein. The other strains, WT and ΔClpX with pUTE-657, were needed as controls to determine normal resistance and loss of resistance to AMPs. Before testing AMP resistance, we first confirmed that the bacterial strains had the pUTE plasmid and the pFtsZ plasmid using PCR and gel electrophoresis. The pUTE primers amplified a smaller region of the plasmid while the pFtsZ primers amplified the inserted FtsZ gene on the plasmid (Figure 1). The PCR products were then examined using gel electrophoresis to confirm proper band size. As expected, the gel showed the strains with the empty pUTE-657 plasmid had a 271 base pair band that appeared at the end of the ladder. The WT pFtsZ strains had FtsZ primers which amplified the inserted FtsZ gene. This was confirmed with the visualization of a 1330 base pair band, which indicates the presence of the FtsZ gene in the pUTE plasmid.

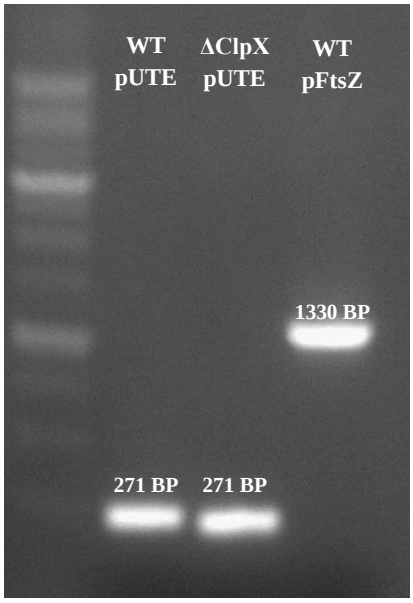

Figure 1.
Confirmation of pUTE-657 and pFtsZ plasmid in *B. anthracis*. Amplification of plasmid DNA showing FtsZ insert in pFtsZ plasmid.

Increased levels of FtsZ with 1mM of IPTG

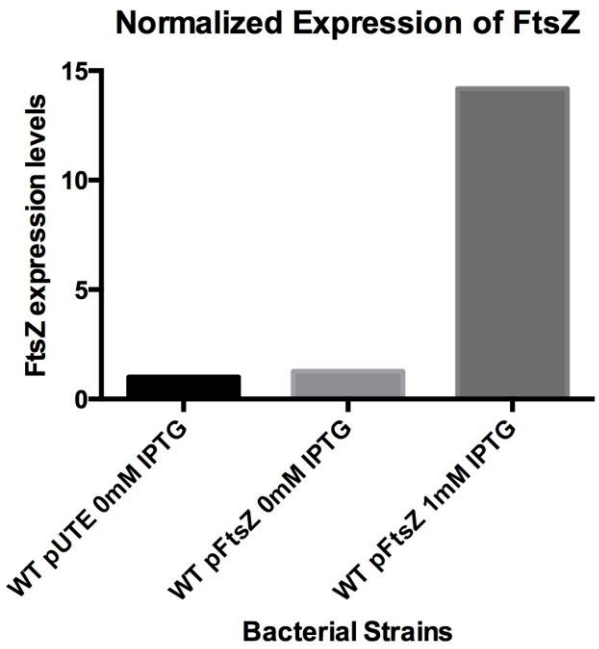

Figure 2
Induction of FtsZ levels occurred at 1mM of IPTG. FtsZ gene expression was measured using QPCR and compared between WT pUTE, WT pFtsZ with 0 mM IPTG and WT pFtsZ with 1 mM IPTG.

Quantitative Real Time PCR (QPCR) was conducted to confirm that the addition of IPTG with the pFtsZ plasmid caused increased expression of FtsZ. RNA was extracted from WT strains containing pUTE-657 with 0 mM of IPTG, pFtsZ with 0 mM of IPTG, and pFtsZ with 1 mM of IPTG and the RNA was then converted to cDNA. The FtsZ gene was then amplified by QPCR using the FtsZ FWD short and REV short primer in each well. As a loading control, GyrB FWD and REV short primers amplified GyrB, a housekeeping gene that was used to normalize FtsZ expression levels. DNA amplification was measured in real time by measuring the amount of fluorescence in each well. The amount of fluorescence present in each well corresponds to the amount of cDNA and ultimately the amount of mRNA present in each strain.

The normalized expression for FtsZ was then calculated using the Livak method with GyrB expression levels. This was to ensure that equal amounts of cDNA were added to each well. After normalization of expression levels, the WT pUTE-657 with 0 mM IPTG showed low levels of FtsZ were present (Figure 2) and provided a baseline of normal FtsZ levels in *B. anthracis*. As expected, the WT pFtsZ 0 mM IPTG also showed similar expression levels as the WT pUTE 0 mM IPTG since IPTG was absent and therefore the pFtsZ plasmid was not induced. Furthermore, the expression of FtsZ in WT pFtsZ with 1 mM of IPTG showed 14 times the normal expression level of FtsZ (Figure 2). Thus, adding 1mM of IPTG was sufficient to induce the pFtsZ plasmid and confirmed the overexpression FtsZ levels in the WT pFtsZ strain.

Determining resistance to antimicrobial peptides

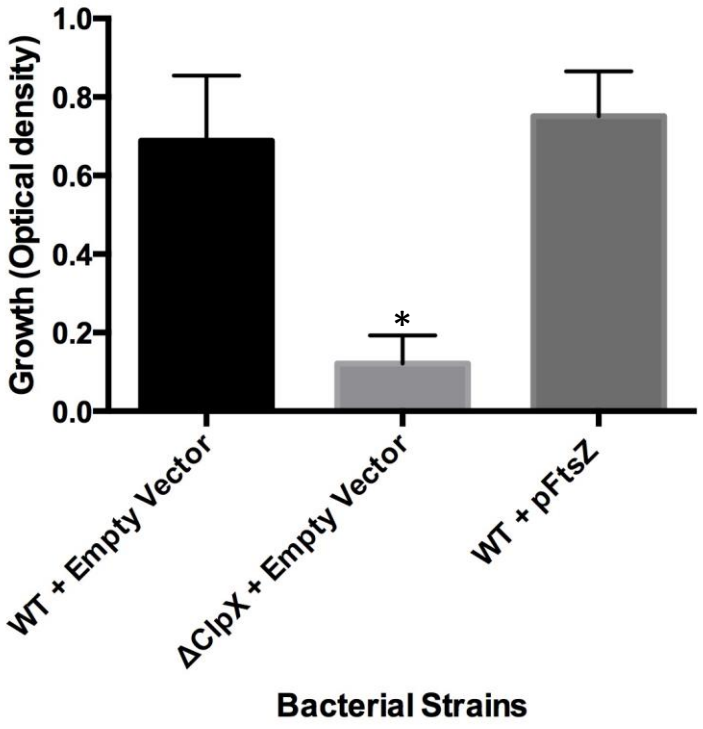

Figure 3

Overexpression of FtsZ does not increase susceptibility to AMPs. Growth after 24 hours of bacterial strains grown in 1mM of IPTG in the presence of 1.6 uM LL-37. Results represented the mean of 3 independent experiments +/- SEM. Asterisk denotes p-value of < 0.05 using one-way ANOVA followed by Tukey's post-hoc test.

After confirming the increased levels of FtsZ by qPCR, we conducted growth assays to determine whether increased levels of FtsZ led to loss of resistance to LL-37, an AMP. After 24 and 48 hours, the optical density (at 600 nm) of each well was measured. WT pUTE and ΔClpX pUTE with 1mM of IPTG were used as controls that indicated normal resistance to AMPs and increased susceptibility to AMPs, respectively. While the addition of IPTG will induce the pUTE-657 vector, the empty vector does not have the inserted FtsZ gene and will not have overexpression of FtsZ. As predicted, ΔClpX pUTE did not grow in 1.6 μM of LL-37 and showed significantly increased susceptibility to LL-37. WT pUTE grew in 1.6 μM of LL-37 and showed normal resistance. Unexpectedly however, WT pFtsZ with 1mM of IPTG showed growth in the 1.6 μM of LL-37 and did not reflect the same result as ΔClpX pUTE.

Long Chains found in WT + pFtsZ Strain

A) **WT pUTE** B) **WT pFtsZ**

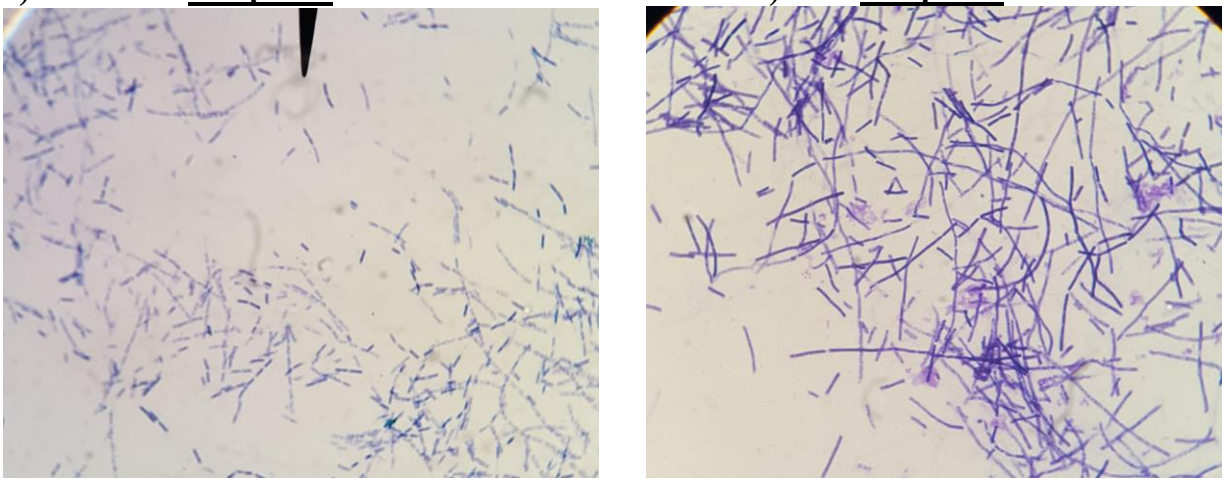

Figure 4.
Overexpression of FtsZ results in increased chain length. Images of WT pUTE (A) and WT pFtsZ (B) were taken with a light microscope under oil immersion at 1000x.

We initially hypothesized that increased susceptibility to AMPs resulted from changes in the cell wall that created extra division sites and mini-cells. Since the growth assay with the FtsZ overexpression did not show increased AMP susceptibility, we next examined the morphology of the bacteria with and without FtsZ overexpression. The three bacterial strains were stained with crystal violet and iodine and examined using a light microscope under oil immersion. Side by side images of each strain showed differences in cell length and chain length (Figure 4). WT pUTE and ΔClpX pUTE strains showed similar results with short cells and short chains (data not shown for the ΔClpX pUTE strains.) The WT pFtsZ showed a greater abundance of long cells and long chains under the same magnification.

DISCUSSION

Previous studies have shown that the ClpX portion of the ClpXP protease is necessary for resistance to AMPs and other antibiotics that target or interact with the cell wall. When visualized with transmission electron microscopy, the ΔClpX mutant also showed aberrant cell division resulting in the formation of mini-cells caused by extra division sites. We proposed that these mini-cells would increase susceptibility of ΔClpX strain due to increase target sites for antimicrobials, many of which act at these division sites. Since it is unlikely the ClpXP protease directly regulates cell division, we believe that the ClpXP protease plays a role in AMP resistance by regulating downstream proteins involved cell division.

In this project, we attempted to understand the resistance mechanism of *B. anthracis* by focusing on a cell division protein, FtsZ, that is normally degraded by the ClpXP protease [13]. Overexpression of FtsZ had previously been seen to induce mini-cell formation in *E. coli* [14]. Therefore, we hypothesized that the increased levels of FtsZ would induce mini-cell formation in *B. anthracis* and show similar loss in resistance to AMPs as the ΔClpX mutant. However, our results show that even when FtsZ is overexpressed, *B. anthracis* had the same level of resistance to the AMP LL-37 as the WT pUTE-657 control. Therefore, these results suggest that increased FtsZ levels are not the cause of loss of resistance in the ΔClpX mutant. However, the retention of resistance seen in the pFtsZ strain may actually be due to lack of mini-cell formation. Our images from the light microscopy imply that overexpression of FtsZ may have had the opposite effect than what we predicted. Increased FtsZ levels may have actually lengthened the cells rather than induced mini-cell formation. This will need to be confirmed in future studies using transmission electron microscopy where the bacterial division sites and cell wall can be visualized in detail.

While overexpression of FtsZ does not increase antimicrobial peptide susceptibility in *B. anthracis*, we still cannot rule out the role of mini-cell formation in antimicrobial peptide resistance. Future studies exploring the role of mini-cell formation in the susceptibility of *B. anthracis* are still needed. One possibility is to focus on other cell division proteins that are dysregulated in ΔClpX and may contribute to mini-cell formation. For example, the YycFG operon has been known to control autolytic activity of bacteria [16] and has also been seen to induce mini-cell formation when over-expressed in *B. subtilis*. [17]. Intriguingly, in *S. aureus* mutant with a deletion of ClpP, yycG was upregulated [18] and in yycG is also up-regulated 2-fold in ΔClpX *B. anthracis* [9]. Therefore, overexpressing the YycFG operon may serve as a better target to induce mini-cell formation in *B. anthracis* and test for antimicrobial susceptibility.

While the connection of FtsZ and ΔClpX did not provide more insight on antibiotic resistance in *B. anthracis*, continuing studies in new proteins such as YycFG are still important in understanding the resistance mechanism of *B. anthracis* and other pathogens. Current concerns in treatment of these microbes have risen due to detection of more antibiotic resistant strains such as methicillin-resistant *S. aureus.* Over the current years, resistance to present antibiotics has developed and continues to increase in many pathogens [18]. However, just as equally concerning, the number of companies developing new antibiotics has declined over the years [19]. These two factors create a scenario for microbes with high resistance to prosper without any effective treatment to combat them. In order to prevent this, current and future research should devote efforts to understanding the resistance mechanism of these pathogens in order to develop novel antibiotic treatments.

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