CONNECTION OF THE ClpXP PROTEASE TO AUTOLYTIC ACTIVITY AND ANTIBIOTIC RESISTANCE IN *BACILLUS ANTHRACIS*

by

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ABSTRACT

The ClpXP protease functions as a global regulator of many bacterial proteins. It is comprised of ClpX, a regulatory ATPase that recognizes and unfolds proteins, and ClpP, which forms the proteolytic core. ClpX and/or ClpP have been linked to virulence in several pathogens including *Bacillus anthracis*. While ClpXP is important for survival within the host, it is unclear the mechanism by which it is doing so. We have found that *B. anthracis* deficient in ClpX (ΔClpX) is more susceptible to antimicrobial agents that interact with the cell wall such as penicillin, daptomycin and cathelicidin antimicrobial peptides but not non-cell wall active agents. Microarray analysis also revealed that expression of the *lrgAB* operon is substantially decreased in ΔClpX and this was confirmed by QPCR. LrgA and LrgB act as negative regulators of autolysis and loss of these increases susceptibility of *Staphylococcus aureus* to penicillin. We hypothesize that the decreased expression of *lrgA/B* in our ClpX mutant leads to increased autolytic activity and a corresponding increase in susceptibility to cell wall-targeting antibiotics. We compared the rate of cell lysis and the activity of autolytic enzymes between wild-type and ΔClpX *B. anthracis*. We found no detectable difference in enzyme activity, but we did observe a small increase in autolytic activity in ΔClpX. We also found that *S. aureus* deficient in LrgA/B is more susceptible to daptomycin. Thus, LrgA/B appears to be involved in resistance to antibiotics besides just penicillin. Therefore, the decrease in *lrgAB* expression in ΔClpX might contribute to the increase in antibiotic susceptibility seen in the ClpX mutant. However, further work is still needed to determine whether there is a direct connection between loss of ClpX, decreased LrgA/B and increased antibiotic susceptibility.

ACKNOWLEDGEMENTS

I would like to acknowledge the support of the University of Texas at Arlington for providing the methicillin-resistant *Staphylococcus aureus* UAMS-1 strains (acquired from Groicher et al.) that have allowed me to conduct my research with other pathogens related to *Bacillus anthracis*. I would also like to thank the Science and Engineering Research Center at Texas Christian University for providing a grant to fund many of the laboratory supplies used to conduct experiments. Finally, I would like to express my gratitude and appreciation for the dedication, support and training that I received from my research mentor, Dr. Shauna McGillivray.

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INTRODUCTION

Bacillus anthracis is a gram-positive, spore-forming soil bacterium and the causative agent of the fatal infection known as anthrax. Anthrax manifests when bacterial endospores enter the body through abrasions in the skin, the gastrointestinal tract or the respiratory tract. Endospores are dormant, reduced forms of vegetative bacteria that can become activated upon entering the body. If inhaled, the activation of these endospores often leads to a systemic infection, characterized by severe bacteremia and toxemia as well as rapid onset of shock and death. Endospores inhaled into the lungs are phagocytosed by resident macrophages and carried to local lymph nodes where the spores germinate into vegetative bacteria and escape from the macrophages. The bacteria multiply in the lymphatic system and express a host of potent virulence factors that subvert the immune system, including toxins and a capsule, an extra-membranous structure that protects bacterial cells from phagocytosis. The resulting toxemia usually causes rapid death in the host. Because of the bacteria's ability to cause such fatal disease, it has been classified as a National Institute of Allergy and Infectious Diseases Category A priority pathogen. It is feared as a potential biological weapon and was most recently used in 2001 to infect U.S. citizens with contaminated mail. Researching the pathogenicity of this bacterium and the causes of its virulence may lead to novel treatment targets and provide greater understanding of host-pathogen relationships of not only *Bacillus anthracis* but other related pathogens (1).

B. anthracis expresses a number of virulence factors that allow it to evade the host immune system and cause death. The most well-characterized of these virulence factors are the toxins, lethal toxin and edema toxin, and a poly-D-glutamate capsule,

which are encoded on the pXO1 and pXO2 plasmids, respectively. Lethal and edema toxin result in massive edema, loss of neutrophil function and release of pro-inflammatory cytokines such as TNF-α and IL-1β that cause the shock and sudden death typically seen in severe anthrax infections (1). In addition, the capsule prevents macrophages from phagocytosing vegetative bacteria, a feature that largely contributes to the bacteremia often seen in many cases (1). Nevertheless, while these virulence factors have been well described, the role of chromosomally-encoded genes in pathogenicity has remained largely unexplored, making them prime targets for further research. Sequencing and analysis of *Bacillus anthracis* Ames (the fully virulent strain) (2) has revealed homologues to a number of chromosomal virulence genes that have already been characterized in the related *Bacillus cereus* and *Bacillus thuringiensis* species (3). Thus, it is likely there are important chromosomally-encoded virulence factors in *B. anthracis* in addition to the plasmid-encoded genes.

With this understanding in mind, my research mentor, Dr. McGillivray, and her colleagues employed a forward genetic screen to identify potential chromosomal genes that might contribute to virulence in *B. anthracis*. This method involves altering an organism's genotype to observe any changes in phenotype. Specifically, they used a transposon-based mutagenesis strategy, which yielded an attenuated mutant with a disruption in a gene called ClpX (4). ClpX is an important component of the intracellular bacterial protease, ClpXP. ClpXP is a global regulator and functions to degrade damaged proteins and regulate the lifespan of many proteins including enzymes and transcriptional regulators. ClpX is responsible for recognition and cleavage of proteins that are then fed into the proteolytic core, which is comprised of ClpP (4). Without ClpX, ClpP is capable

only of recognizing and degrading peptides fewer than 7 amino acids and thus ClpX serves as an integral component of the protease's function (5). The genetic knockout of ClpX in *B. anthracis* Sterne (an attenuated strain lacking the pX02 plasmid and therefore the capsule synthesis genes) revealed that ClpX is a potent virulence factor. In support of this, *B. anthracis* deficient in ClpX (ΔClpX) were unable to cause infection and were also more susceptible to cathelicidins, an antimicrobial peptide important in host defense (4).

These findings confirmed the possibility that ClpX represents a novel virulence factor for *B. anthracis* infection. However, the mechanism by which ClpX increases the bacteria's virulence remains a topic for further investigation. To explore possible ways in which ClpX might be functioning, a microarray was performed to examine differences in gene expression between wild-type and ΔClpX strains. Five thousand genes were screened, and we found that about 150 were up 3-fold in wild-type *B. anthracis* versus ΔClpX and 200 were up 3-fold in ΔClpX versus wild-type. Although many of these genes would be interesting to pursue, my project will focus on two genes called *lrgA* and *lrgB*, which are regulated together on the same operon. These genes were down-regulated 6-fold in the ClpX mutant relative to wild-type and this was confirmed using quantitative polymerase chain reaction (QPCR) (unpublished data). Previous research in *Staphylococcus aureus* has indicated that the LrgAB gene products play a role in bacterial cell wall metabolism. These studies revealed that loss of *lrgAB* results in increased autolysis, a tightly controlled process by which bacteria partially degrade their own cell walls to allow for expansion and growth. Specifically, it is believed that LrgA functions as a negative regulator of autolysis by preventing export of murein hydrolases, the enzymes that catalyze autolytic activity (6). As a result, if LrgA/B were not present to

mitigate export of these enzymes, it is possible that autolytic activity would increase and the cell wall would become weakened and more susceptible to cell wall-targeting agents. In support of this hypothesis, *S. aureus* mutants lacking *lrgAB* exhibit increased penicillin-induced killing of cells. Furthermore, other studies with *S. aureus* reveal that when the bacteria are deficient in other negative regulators of autolysis, they become more susceptible to cell wall-active antibiotics such as vancomycin (7).

Loss of ClpXP in *B. anthracis* also results in greater vulnerability to cell wall-interacting antibiotics such as penicillin, vancomycin and daptomycin (8). In *S. aureus*, resistance to all three of these antibiotics has been linked to autolysis or autolytic enzymes in some way (9, 10). Therefore, we hypothesize that loss of ClpX is leading to increased autolytic activity, which is causing increased susceptibility to antibiotics that target the cell wall. We hypothesize this is mediated via LrgA/B in *B. anthracis.*

METHODS

Bacterial Strains and Culture Conditions

Bacillus anthracis Sterne strain ($pXO1^{+}pXO2^{-}$) was grown in Brain-Heart Infusion (BHI) medium and methicillin-resistant *Staphylococcus aureus* UAMS-1 was grown in Tryptic Soy Broth (TSB) at 37 °C under aerobic conditions.

Construction of *B. anthracis* Sterne *lrgA* insertional mutant

The *B. anthracis* LrgA gene sequence was amplified via PCR from genomic DNA using the following primes: LrgA IM EcoRI Fwd 5'-agtgaattcgcaaagtttcatg-3' and LrgA IM HindIII Rev 5'-actaagcttcgattaccgatg-3'. The PCR product was collected, purified using the gel purification kit (Qiagen) and inserted into pGEM-T Easy vector. The LrgA insert was then subcloned into pHY304 using the restriction enzymes EcoRI and HindIII.

The construct was transformed into MC1061 *E. coli* and grown at 30 °C for 48 hours. Bacteria containing the construct and insert were selected for by growing the bacteria on BHI agar plates containing 500 μg/ml erythromycin. The construct was then purified from MC1061 *E. coli* cells using the plasmid miniprep kit (Qiagen) and transformed into GM2163 *E. coli*, a methylation deficient strain, and grown at 30 °C for 48 hours. Bacteria containing the construct were selected for in the same ways as for MC1061 *E. coli.* The construct was purified using the same kit and transformed into *B. anthracis* Sterne. Bacteria were grown at 30 °C for 48 hours on 500 μg/ml erythromycin BHI plates to select for cells containing the construct. Seven colonies were selected from these plates and the presence of the plasmid was assessed by colony PCR using the following primers: pHY3065 Fwd 5'-acgactcactatagggcgaattgg-3' and pHY3175 Rev 5'-ccgctctagaactagtggatcccc-3'. Bacteria containing the plasmid were propagated overnight at 30 °C in liquid BHI + 5 μg/ml erythromycin. After 16 hours, 100 μl of bacteria were passaged into 5 mls of BHI + 5 μg/ml erythromycin and grown at 37 °C. After 8 hours, bacteria were diluted 1:500, 1:1000, and 1:5000 and 100 μl of each dilution was plated on BHI agar plates containing 5 μg/ml erythromycin. Plates were incubated for 16 hours at 37 °C to select for bacteria with plasmid integration. Seven colonies from these plates were selected and integration was confirmed with colony PCR using the following primers: pHY3175 Rev 5'-ccgctctagaactagtggatcccc-3' and LrgA Rev 5'-3'.

Minimum Inhibitory Concentrations Assays

Wild-type, ΔLrgAB, and ΔLrgAB + pLrgAB *S. aureus* (6) were propagated overnight for 16 hours and then diluted 1:20 and grown to early log phase at an optical density of 0.4 at 600 nm wavelength. Cultures were centrifuged for 10 minutes at 4000 x

g, suspended in an equivalent volume of phosphate buffered saline (PBS) and diluted 1:500 in Mueller Hinton Broth II (MHBII) + 50 µg/ml Ca^{2+} and 10 µg/ml Mg^{2+}. Solutions of daptomycin at 24 µg/ml, 16 µg/ml, 12 µg/ml and 8 µg/ml were then prepared in the same medium. One hundred µl of each concentration of daptomycin was added to 100 µl of 1:500 diluted bacteria in a 96-well plate to dilute bacteria 1:1000 and daptomycin to final concentrations of 12 µg/ml, 8 µg/ml, 6 µg/ml and 4 µg/ml. The plate was incubated overnight for 16 hours at 37 °C. Percent growth was calculated by measuring the optical density of each well at 600 nm wavelength and normalizing it to the 0 µg/ml control well.

Wild-type, ΔLrgAB, and ΔClpX *B. anthracis* were propagated overnight and grown to early log phase at an optical density of 0.4 at 600 nm wavelength. Cultures were centrifuged for 10 minutes at 4000 x g, suspended in an equivalent volume of phosphate buffered saline (PBS) and diluted 1:50 in RPMI + 5% LB. Solutions of LL-37 at 2.4 µg/ml, 2 µg/ml, 1.6 µg/ml and 1 µg/ml were then prepared in the same medium. One hundred µl of each concentration of LL-37 was added to 100 µl of 1:50 diluted bacteria in a 96-well plate to dilute bacteria 1:100 and LL-37 to final concentrations of 1.2 µg/ml, 1 µg/ml, 0.8 µg/ml and 0.5 µg/ml. The plate was incubated overnight at 37 °C. Percent growth was calculated by measuring the optical density of each well at 600 nm wavelength and normalizing it to the 0 µg/ml control well.

Extraction of RNA, Preparation of cDNA and Assessment of LrgA Expression

Cultures of wild-type *B. anthracis* were grown overnight for 16 hours at 37 °C in 24 µg/ml, 12 µg/ml, 6 µg/ml and 0 µg/ml penicillin. Cultures were lysed by pulsing 2 x 45 s in a mini-bead beater. RNA was purified using the RNAeasy kit (Qiagen) followed by

DNase treatment using the Turbo DNase-free kit (Ambion). Equal amounts of RNA were reverse transcribed using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). QPCR was performed using SYBR Green master mix (Life Technologies) and the following primers: FusA Fwd 5'-aagctggtggtgctgaagcac-3'; FusA Rev short 5'-ccatttgagcagcaccagtga-3'; LrgA Fwd 5'-ccaattccaatgccctcatc-3'; LrgA Rev short 5'-ctgaaatacctgatgggacg-3'.

Analysis of QPCR

LrgA expression at each concentration of penicillin was compared to LrgA expression in bacteria grown without penicillin. cDNA was analyzed in triplicate for LrgA expression and FusA expression, a housekeeping gene unaffected by penicillin exposure. The average Ct value of the three wells was used to normalize LrgA expression to FusA. The Ct value of FusA grown at a particular concentration of penicillin was subtracted from the Ct value of FusA grown at 0 μg/ml penicillin, yielding ΔCtFusA. The same calculation was used for LrgA to yield ΔCtLrgA. ΔCtLrgA was then subtracted from ΔCtFusA. The solution of 2 to the power of this value was used as the normalized expression ratio for LrgA.

CtFusA (PCN X) – CtFusA (PCN 0) = ΔCtFusA

CtLrgA (PCN X) – CtLrgA (PCN 0) = ΔCtLrgA

(ΔCtLrgA - ΔCtFusA) = ΔΔCtLrgACtFusA

$2^{-\Delta\Delta LrgAFusA}$ = normalized expression ratio

Preparation of lyophilized bacterial cells

A 200-ml culture of wild-type *B. anthracis* was grown overnight for 16 hours, autoclaved and centrifuged at 4000 x g. The pellet was resuspended in PBS, divided into

1-ml aliquots and vacufuged for 8-10 hours. Dried bacterial cells were then ground into a fine powder and stored at 4°C.

Zymography

Autolysins were extracted from 50-ml cultures of wild-type and ΔClpX *B. anthracis* grown to an optical density of 0.4 at 600 nm wavelength. The cultures were centrifuged at 4000 x g and the cell pellets were resuspended in 500 µl of SDS-PAGE sample buffer (1% [wt/vol] SDS, 1 mM EDTA, 10% [vol/vol] glycerol, 5% [vol/vol] β-mercaptoethanol, 0.0025% [wt/vol] bromophenol blue, and 50 mM Tris-HCl [pH 7.5]). Autolysins were harvested by boiling the suspension for 3 minutes at 100°C, centrifuging at 15,000 x g for 5 minutes and collecting the supernatant. Extracts were run on an SDS-PAGE gel prepared with 10 mg/ml of autoclaved, lyophilized bacterial cells for 1 hour at 120 V. Gels were soaked for 30 minutes with gentle agitation in ultrapure DI water at room temperature. The gels were then transferred to 250 ml of renaturation solution (0.1% Triton X-100, 10 mM $MgCl_2$, and 25mM Tris-HCl [pH 7.5]) and allowed to incubate for 30 minutes at room temperature with gentle agitation. The gels were then transferred to 250 ml of the same solution and allowed to incubate 16 hours at 37 °C with gentle agitation. The gels were then rinsed in distilled water, stained in 0.1% methylene blue in 0.01% KOH for 3 hours, and destained in ultrapure DI water.

Coomassie Protein Assay

Autolysins extracted for the zymography gel were run on a plain SDS-PAGE gel. The gel was then stained with Simply Blue Coomassie G-250 for 45 minutes and then rinsed three times in ultrapure DI water at 5 minute intervals to remove the dye.

Autolysis Assay

Overnight cultures of wild-type and ΔClpX *B. anthracis* were grown to early log phase at an optical density of 0.4 at 600 nm wavelength and stationary phase (16 hours of growth). Bacteria were pelleted at 4000 x g for 10 minutes, washed in PBS, and resuspended in PBS to create a final concentration of 10x. Penicillin was prepared at 200 μg/ml, 100 μg/ml and 50 μg/ml. One hundred μl of 10x bacteria were added to 100 μl of penicillin at each concentration in a 96-well plate. Each well contained final concentrations of 5x bacteria and 100 μg/ml, 50 μg/ml or 25 μg/ml penicillin. Bacteria were incubated at room temperature and the optical density of each well was assessed at 600 nm wavelength at 30-minute intervals for 4 hours.

Statistics

GraphPad Prism was used to generate graphs. Results depicted in each graph represent the mean ± standard deviation of at least 3 different experiments. QPCR analysis of cDNA was performed in triplicate each time. In addition, the rate of autolytic activity was assessed in triplicate at each concentration of penicillin. Significance of data obtained from the autolysis assay was determined using a *t*-test. Significance of data obtained from the minimum inhibitory concentration assays was assessed using a one-way ANOVA test and Tukey's post hoc test.

RESULTS

To determine if *S. aureus* deficient in LrgAB (ΔLrgAB) was more susceptible to cell wall-interacting antibiotics, we incubated wild-type and ΔLrgAB in increasing amounts of daptomycin overnight and then measured the amount of growth. Growth of ΔLrgAB *S. aureus* is inhibited at lower concentrations of daptomycin than wild-type (Fig. 1A). This finding was statistically significant and conclusive with our hypothesis that

lack of negative regulators of autolysis, such as LrgAB, disrupt cell wall integrity and lead to increased vulnerability to cell wall-active agents. However, only partial restoration of the wild-type phenotype was observed with the complemented strain, that is ΔLrgAB that have been transfected with a plasmid encoding the *lrgAB* genes (Fig. 1B). It is possible that these genes are under the control of a certain promoter or regulated by specific enhancer sequences in the *S. aureus* genome that are not present in the plasmid vector. If so, the genes would not be fully expressed in the complemented strain and a complete return to the wild-type phenotype would not occur. Nevertheless, these findings suggest that *lrgAB* are critical genes for bacterial survival and their complete absence renders the bacteria more vulnerable to cell wall-active agents.

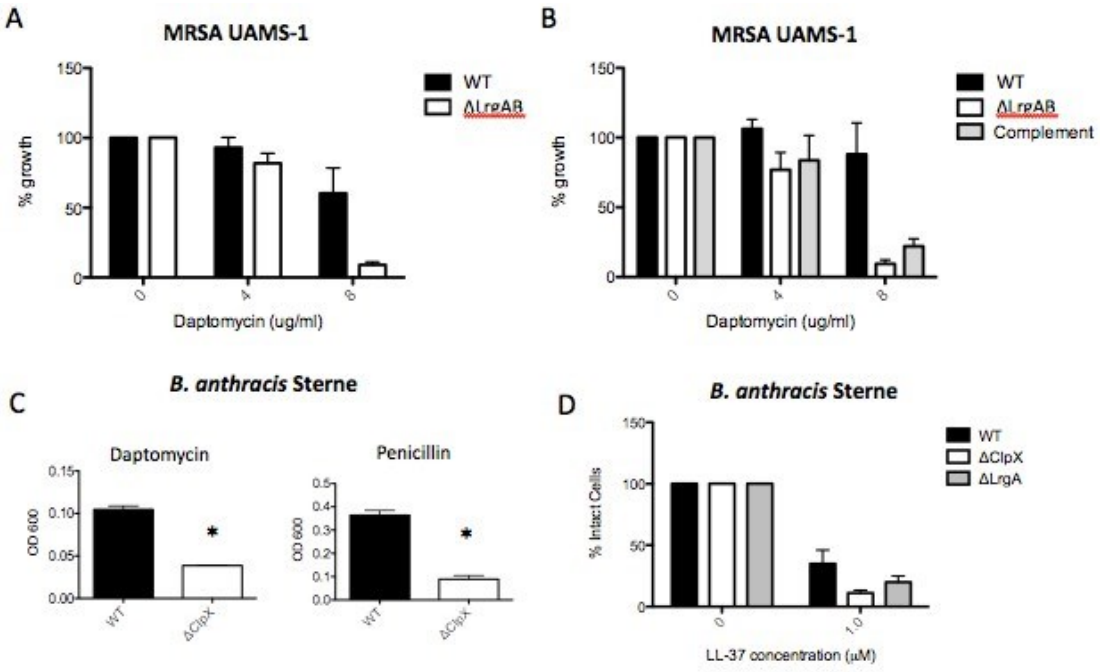

FIG 1 Determination of the minimum inhibitory concentration of daptomycin for MRSA UAMS-1 and *B. anthracis* Sterne; (A) Wild-type and ΔLrgAB and (B) wild-type, ΔLrgAB, and ΔLrgAB + pLrgAB *S. aureus* were grown to early log phase (OD 0.4 at 600 nm wavelength), diluted 1:1000 and grown in the absence of antibiotic and at 4 and 8 μg/ml daptomycin; (C) Wild-type and ΔClpX *B. anthracis* were grown in the presence and absence of daptomycin and penicillin and optical density was measured to assess growth; (D) Wild-type, ΔClpX and ΔLrgA *B. anthracis* were grown in the presence and absence of antimicrobial peptide LL-37 and optical density was measured to assess growth. Mean results ± standard deviation from at least 3 separate experiments performed in triplicate are shown.

Interestingly, this same finding was observed with *B. anthracis* deficient in ClpX, which were also more susceptible to daptomycin, among other cell wall-active antimicrobials (Fig. 1C). Because lack of ClpX in *B. anthracis* and lack of LrgAB in *S. aureus* results in similar phenotypes, it is possible that the biochemical pathways involving ClpX and LrgAB are connected. Moreover, *B. anthracis* deficient in LrgA do not grow as robustly as wild-type when exposed to the antimicrobial peptide, LL-37 (Fig. 1D). This hypothesis is further substantiated by the fact that *B. anthracis* deficient in ClpX also exhibits a decrease in LrgA expression (Fig 2) (unpublished data). Because this attenuated phenotype exhibits greater susceptibility to agents that target the cell wall, it is likely that ClpX and LrgA play a role in cell wall regulation or metabolism. LrgA has already been well characterized in *S. aureus* as a negative regulator of autolytic activity, but ClpX has not been well studied in relation to autolysis or other cell wall-associated processes.

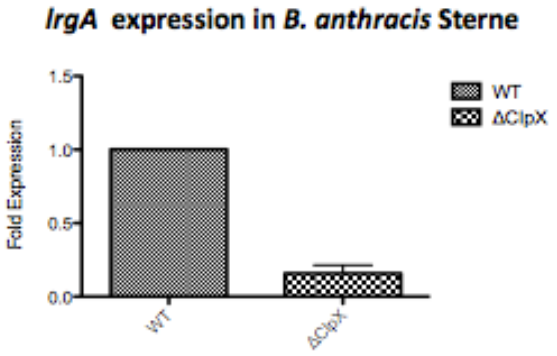

FIG 2 Expression of *lrgA* in wild-type and ΔClpX *B. anthracis* was assessed by extracting RNA from bacterial cells, reverse-transcribing it to cDNA and quantifying gene expression with QPCR.

To determine a role for ClpX in the regulation of autolytic activity, we incubated wild-type and ΔClpX *B. anthracis* in various concentrations of penicillin and measured

the rate of cell lysis over a period of several hours. We found that while *B. anthracis* deficient in ClpX exhibited a slightly greater rate of penicillin-induced autolysis, there is no statistically significant difference in activity between the mutant and wild-type strains (Fig 3A). Moreover, this slight difference only appears in *B. anthracis* grown to log phase, an exponential phase of bacterial cell growth where all cells are dividing at a constant rate. Stationary phase bacteria do not exhibit any difference in autolysis (Fig 3D). This finding possibly reflects the temporal expression of genes involved with regulation of autolytic activity at log phase, but not stationary phase at which point the accumulation of certain metabolites might be inhibitory to cell growth and expression of noncritical genes. In addition, increasing the concentration of penicillin did not significantly change the degree to which ClpX mutants responded to penicillin-induced autolysis (Fig 3B, C).

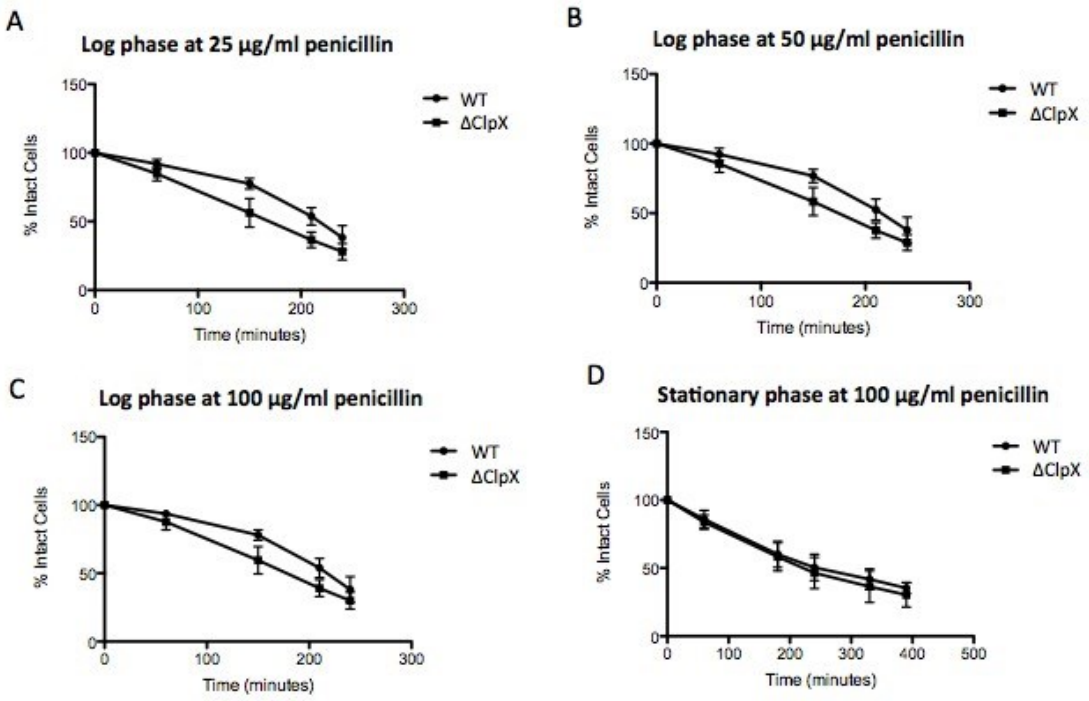

FIG 3 Rate of penicillin-induced autolysis in wild-type and ΔClpX *B. anthracis* Sterne; (A, B, C) Bacteria were grown to early log phase (OD 0.4 at 600 nm wavelength) and incubated for 4 hours in 25, 50, and 100 µg/ml PCN, respectively; (D) Bacteria were grown for 16 hours to stationary phase and incubated in 100 µg/ml penicillin for 6.5 hours. Mean results ± standard deviation from 3 separate experiments performed in triplicate are shown.

We expected ΔClpX *B. anthracis* to exhibit a greater rate of autolysis than we observed, mainly because of their strong sensitivity to cell wall-active agents. However, since the ClpX mutant does lyse slightly faster than wild-type bacteria, it is not implausible that autolytic activity is somewhat less controlled in the mutant bacteria. Because this possibility could not be confirmed by observing autolysis on a macroscopic level, we next determined if there was any difference in the activity of the autolytic enzymes (autolysins) themselves.

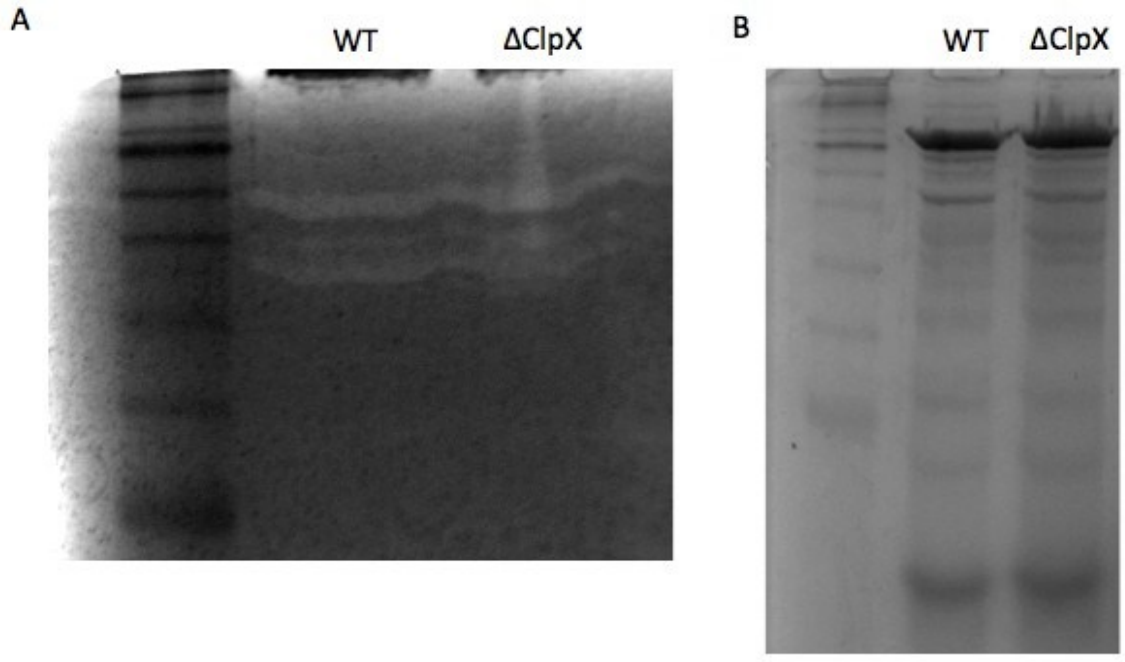

FIG 4 Enzymatic activity of intracellular autolysins harvested from wild-type and ΔClpX *B. anthracis* Sterne. (A) Autolysins were extracted from bacteria grown to early log phase (OD 0.4 at 600 nm wavelength) and run on an SDS-PAGE gel embedded with autoclaved, lyophilized cell walls. Zones of clearing indicate areas where autolysins have degraded cell wall extracts; (B) Coomassie protein assay; autolysins were separated on a SDS-PAGE gel and stained with Simply Blue Coomassie G-250 to show that equal amounts of protein were added to each well in the zymography gel.

Because only log phase bacteria exhibited a difference in the rate of autolysis, we chose not to extract autolysins from stationary phase bacteria. We harvested autolysins from both wild-type and ΔClpX *B. anthracis* grown to log phase by boiling the bacterial cultures in a sample buffer, centrifuging, and then collecting the supernatant. The

enzymes were then run on an SDS-PAGE gel embedded with lyophilized bacterial cell walls. Autolysins function to degrade bacterial cell walls, which are made of a rigid peptidoglycan matrix that must be broken to allow for expansion and growth. The zones of clearing in the gel indicate digestion of the cell wall extracts by the autolytic enzymes. Our gel confirms that the enzymes are active, but there appears to be no difference in the activity of enzymes extracted from wild-type compared to the ClpX mutant (Fig. 4A). In addition, the Coomassie Protein Assay confirms that equal amounts of enzyme were added to each well in the SDS-PAGE gel (Fig. 4B). Thus, while there might be a slight increase in autolytic activity in ΔClpX *B. anthracis* (Fig. 3A, B, C), this difference is only detectable on a macroscopic level. LrgA/B are known to be downregulated in the ClpX mutant, but it is possible that even more dampening of LrgA/B expression or perhaps full silencing is needed to significantly alter autolysis or autolytic enzyme activity. In addition, an uncontrolled increase in autolytic activity would be detrimental to a healthy bacterial cell since it would compromise the integrity of the cell wall. As a result, it is likely that downregulation of LrgA/B alone is not sufficient to undermine completely the process of autolysis. In addition, while *lrgA/B* has been characterized as an autolytic regulatory gene in *S. aureus*, its function and activity in *B. anthracis* is unknown. Thus, *lrgA/B* may not function in exactly the same way as in *S. aureus*.

To elucidate a role for LrgA in *B. anthracis*, we grew wild-type bacteria overnight in the presence of various concentrations of penicillin. We then extracted RNA from these cultures, reverse transcribed it to cDNA, and used QPCR to assess if LrgA gene expression increased as the concentration of penicillin increased. Penicillin was used to create an environment stressful to the cell wall and thus to encourage expression of LrgA.

Because LrgA has been shown to suppress autolytic activity in *S. aureus*, we hypothesized that its expression would increase in the presence of harsh conditions to prevent self-degradation of the cell wall. We found that LrgA does not fluctuate in a predicable way. Rather, its expression appears to decrease, increase, and then decrease again as antibiotic concentrations increase (Fig 5). The degree to which expression shifts, however, is small and not statistically significant.

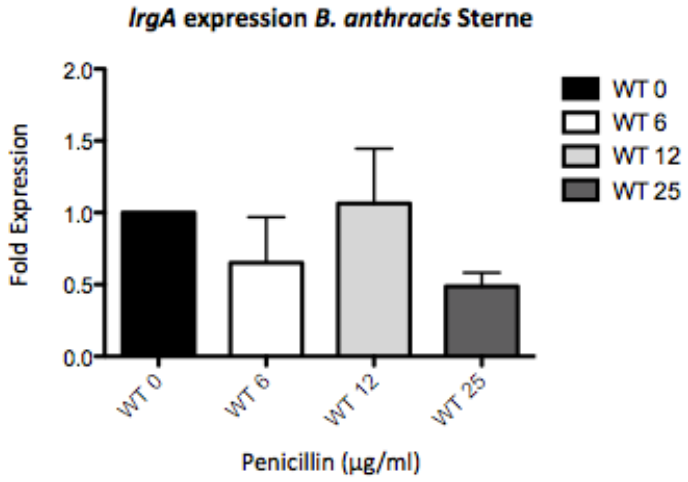

FIG 5 Changes in the expression of *lrgA* in wild-type *B. anthracis* Sterne when exposed to increasing concentrations of penicillin. RNA was extracted from wild-type cultures grown for 16 hours, was reverse-transcribed to cDNA, which was subjected to QPCR analysis to quantify gene expression.

DISCUSSION

While we have not elucidated a full mechanism for the attenuated phenotype observed in ΔClpX *B. anthracis*, we have uncovered several factors that might contribute to decreased virulence. Microarray analysis has revealed that expression of the *lrgAB* operon is substantially decreased in ΔClpX and this was confirmed by QPCR. Our hypothesis suggests that LrgA, as a negative regulator of autolysis, should increase in response to cell wall stress in wild-type bacteria. In attempting to characterize a role for

LrgA in cell wall maintenance, however, we were unable to detect if LrgA is transcribed in response to this kind of stress. Even though our methods did not reveal any clear increase in LrgA expression, it is still possible that increased expression does occur. LrgA may be regulated at the protein level rather than at the RNA level, in which case any changes in expression would not be observed with QPCR. In addition, increased LrgA expression may only be beneficial to a certain point, perhaps at a concentration of penicillin lower than those we used, after which it becomes too inhibitory for cell growth and development. Further studies are needed to fully assess the role of LrgA in autolysis in *B. anthracis*.

Regardless, *lrgA* has been well characterized as an autolytic regulatory gene in *S. aureus*, and loss of LrgA/B increases susceptibility of *S. aureus* to antibiotics that interact with the cell wall, such as penicillin (6) and daptomycin, but not non-cell wall active agents. In addition, loss of ClpX in *B. anthracis* also increases susceptibility to penicillin and daptomycin. Thus, loss of LrgAB in *S. aureus* causes increased vulnerability to the same cell wall-targeting agents as loss of ClpX in *B. anthracis*. Furthermore, loss of LrgA in *B. anthracis* also causes increased susceptibility to these agents. Because the ΔLrgA *B. anthracis* mutant is not as attenuated as the ΔClpX *B. anthracis* mutant or the ΔLrgAB *S. aureus* mutant, it is likely that disruption of LrgB is also required to produce a significantly weakened phenotype. In support of this, LrgB is fully silenced in the ΔLrgAB *S. aureus* mutant and expressed at very low levels in the ΔClpX *B. anthracis* mutant. Nevertheless, the partially attenuated phenotype observed in ΔLrgA *B. anthracis* suggests a promising trend that the *lrgAB* operon is involved in virulence.

These data indicate that loss of these genes may disrupt the integrity of the cell wall. Accordingly, we have found that *B. anthracis* deficient in ClpX exhibits a slight increase in autolytic activity, which might lead to excessive cell wall damage. It is possible that this increase in autolysis is caused by the suppression of LrgA/B as observed in ClpX mutants. However, further suppression or even full silencing of LrgAB may be required to alter autolysis significantly. In addition, disruption of other genes involved in the regulation of autolysis may be necessary before such an essential cellular metabolic process is undermined.

Our data indicate a possible mechanism that clarifies the connection between loss of ClpX, decreased expression of LrgAB, and increased susceptibility to cell wall-interacting antibiotics. Because the ClpXP protease is a global regulator of various proteins in the cell, including enzymes and transcription factors, it is possible that it controls the lifespan of a particular repressor that binds to the LrgAB operator and ensures the repressor's regular degradation. Without the ClpX unit, the protease is only capable of recognizing and degrading peptides fewer than 7 amino acids (5). Thus, in a ClpX mutant, a repressor to the *lrgAB* operon would remain intact and actively suppress LrgAB expression. Because LrgA/B function as negative regulators of autolysis, their absence in the cell could trigger uncontrolled autolytic activity and in turn damage the cell wall. This damage might then expose the cell wall to antibiotic attack. Although many aspects of this scheme are yet to be proven definitively, our data provide encouraging evidence for further work in characterizing the link between loss of ClpX, decreased levels of LrgA/B and increased antibiotic susceptibility. Determining the

mechanism through which loss of ClpX attenuates the bacteria will increase the variety of therapeutic targets for infection with *B. anthracis* or other related pathogens.

Since the discovery of antibiotics, bacterial resistance has followed only shortly behind. Resistance continues to rise as pathogens such as methicillin-resistant *Staphylococcus aureus* (MRSA) and extreme drug resistant tuberculosis (XDR-TB) become even greater threats to worldwide clinical medicine. These pathogens have become increasingly resistant to treatment partly because most antibiotics derive from a very small set of molecular scaffolds for which a growing number of bacteria have acquired near total resistance (11). It is essential that we focus research on finding new, unexplored sources of potential antimicrobials that exert their effects in a completely novel fashion.

The ClpXP protease, the primary focus of our lab's research, represents a perfect target for new antibiotics both because of its critical role in virulence among multiple bacterial pathogens and its conserved nature (5, 4). Targeting virulence factors is a novel strategy in antibiotic development. Instead of directly killing bacteria, the pathogen is disabled thus allowing for easier clearance by the host immune system (12). This approach has been successfully applied in animal models of infection for other virulence factors such as the golden-pigment of *S. aureus*, which protects against reactive oxygen species (13). With regard to ClpX, the gene is not only essential for virulence in several bacterial pathogens, but it is also necessary for resistance to several clinically important classes of antibiotics (8). Use of a ClpXP inhibitor could therefore cripple a pathogen's disease-causing abilities while simultaneously making it more susceptible to antibiotic treatment. ClpXP is also a highly conserved protease among bacteria (14). An inhibitor

designed against ClpXP in *E. coli* was also found to be effective in both *B. anthracis* Sterne and *S. aureus* and the phenotypes seen in *B. anthracis* (increased sensitivity to cathelicidin antimicrobial peptides and daptomycin) were also seen in *S. aureus* (8). Thus, a drug targeting ClpXP could be useful for a variety of bacterial infections.

Although ClpXP clearly plays an important role in the cell and has been investigated as a potential drug target (4, 15, 16) much is still not understood regarding its functions. This study has provided new insight into some of the mechanistic details of the activity of ClpXP in the cell. We have described a possible link between increased autolytic activity and antibiotic resistance, and we have suggested that this connection could be mediated by the LrgA/B proteins. Furthermore, we have shown that these proteins are associated with the ClpXP protease in some way. Further work is needed to determine if other genes involved in autolysis might play a role in the attenuation of ClpX mutants and to assess whether these genes may act with LrgA/B synergistically to cause virulence in *B. anthracis*.

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