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The Role of SigM and GlpF on Cell Wall Active Antibiotic Susceptibility in *Bacillus anthracis* Sterne

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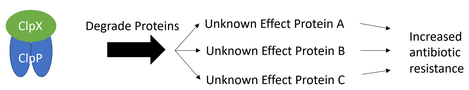
Abstract

The bacterium *Bacillus anthracis*, the causative agent of anthrax, possesses several genes that contribute to virulence. Our lab had previously discovered that chromosomal gene *clpX* is essential for *B. anthracis* virulence. ClpX is an ATPase that is part of the ClpXP proteasome found in many bacteria. Loss of ClpX in *B. anthracis* Sterne results in increased susceptibility to cell wall targeting antibiotics like penicillin and daptomycin. However, the mechanism behind ClpX's role in antibiotic resistance is not well understood and it is likely that multiple pathways are affected by the loss of this global protease. We recently conducted a microarray to find which genes are up or down regulated in ΔClpX compared to wild-type (WT) *B. anthracis*, with several of the 119 found genes connected to cell-wall active antibiotics like penicillin. In this study, we focused on three of these genes: *msrA*, *glpF*, and *sigM*. We confirmed the microarray, showing that *msrA*, *glpF*, and *sigM* gene expression in ΔClpX strains significantly differs from wild-type *B. anthracis* Sterne via QPCR. *glpF* and *sigM* insertional knockout mutants were made to test whether these genes were necessary for antibiotic resistance. Removing *sigM* increased penicillin susceptibility, but no conclusive results for daptomycin. We tested the mutants' virulence in our invertebrate animal model *G. mellonella* and found notable decreases in virulence for ΔSigM and ΔGlpF. SigM appears to also mediate antibiotic resistance and may at least partially account for the loss of resistance seen in the ΔClpX mutant. Further studies need to confirm their exact role in mediating antimicrobial resistance and potentially virulence for *B. anthracis.*

Background Info

Anthrax is a zoonotic disease transmitted to humans through contact with infected animals. It can be fatal depending on the method of entry and even with treatment [1, 2]. The major virulence factors for *B. anthracis* are its two plasmids, pXO1 and pXO2 [1]. One virulence factor outside of the plasmids is the ClpX protein, which serves as part of the ClpXP proteolytic complex which plays a role in protein folding, activation, or disaggregation [3, 4]. Previous work in our lab found that losing the *clpX* gene increased susceptibility to cell-wall active antibiotics like penicillin and daptomycin [5, 6]. The mechanism behind this action was not understood



In order to know which genes were affected by *clpX*, global gene expression for wild-type *B. anthracis* was compared to ΔClpX using a microarray [6]. Of the significantly up or down regulated genes, several were related to the cell wall, and could contribute to cell-wall active antibiotic resistance. *lrgAB* impacted antibiotic resistance, but not as much as *clpX*, indicating more genes were related. This study took three other identified genes, *sigM, glpF,* and *msrA*, and confirmed that they were regulated by ClpX. Insertional knockout mutants were then created for *sigM* and *glpF* and penicillin and daptomycin minimum inhibitory concentration (MIC) assays and *G. mellonella* animal model virulence assays were run on the mutants.

References

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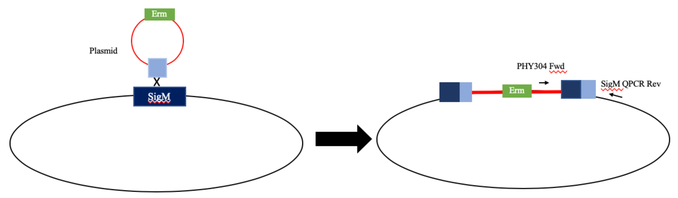
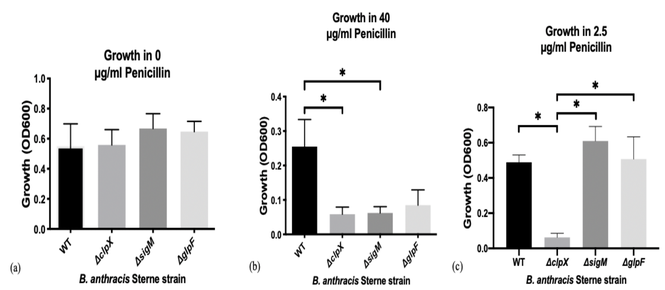
3. McGillivray, S.M., et al., *ClpX Contributes to Innate Defense Peptide Resistance and Virulence Phenotypes of Bacillus anthracis.* Journal of Innate Immunity, 2009.**1**(5): p. 494 506.

Creating *sigM* and *glpF* Insertional Mutants



The pHY304 plasmid (red circle) was inserted into the WT *B. anthracis* Sterne's chromosome (black circle) using homologous recombination. Primers used to confirm integration are shown by the black arrows.

Penicillin and Daptomycin MIC Assays

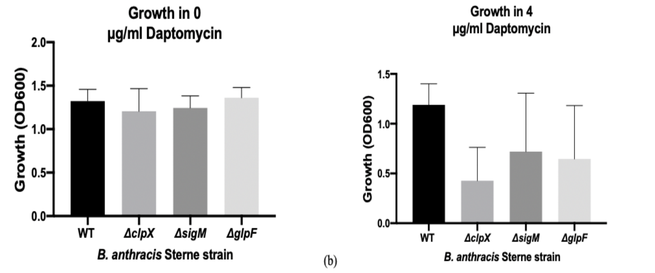


Growth of *B. anthracis* Sterne strains WT, ΔClpX, ΔSigM, and ΔGlpF in an overnight penicillin MIC assay measured at (a) 0 ug/ml penicillin, (b) 40 ug/ml penicillin, and (c) 2.5 ug/ml penicillin. Statistically significant differences demonstrated by * indicating p<0.05 via a one-way ANOVA with Tukey-Kramer *post hoc* analysis. Data represents mean of N = 4 trials with error bars representing standard deviation.

At 40 µg/ml, ΔSigM was statistically different from WT, whereas ΔGlpF was not. However, at 2.5 µg/ml, ΔClpX was inhibited significantly from WT but the two mutants were not. This indicates that while *sigM* contributes to antibiotic resistance given the susceptibility to penicillin when removed, it is not as influential as *clpX* is overall.

References

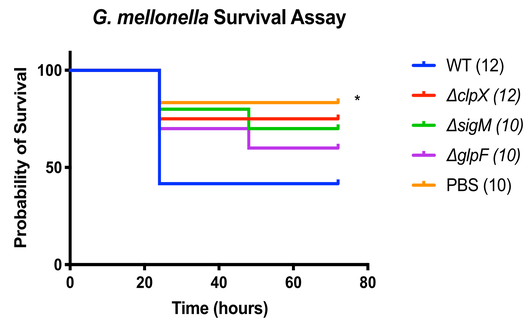
4. Frees, D., et al., *Clp ATPases and ClpP proteolytic complexes regulate vital biological processes in low GC, Gram-positive bacteria.* Molecular Microbiology, 2007. **63**(5): p. 12851295.
5. McGillivray, S.M., et al., *Pharmacological Inhibition of the ClpXP Protease Increases Bacterial Susceptibility to Host Cathelicidin Antimicrobial Peptides and Cell Envelope-Active Antibiotics.* Antimicrobial Agents and Chemotherapy, 2012. **56**(4): p. 1854-1861
6. Claunch, K.M., et al., *Transcriptional profiling of the clpX mutant in Bacillus anthracis reveals regulatory connection with the lrgAB operon.* Microbiology-Sgm, 2018.**164**(4): p. 659-669.
7. Brotz-Oesterhelt, H. and P. Sass, *Bacterial caseinolytic proteases as novel targets for antibacterial treatment.* International Journal of Medical Microbiology, 2014. **304**(1): p. 23-30.



Growth of *B. anthracis* strains WT, ΔClpX, ΔSigM, and ΔGlpF in an overnight daptomycin MIC assay measured at (a) 0 ug/ml daptomycin and (b) 4 ug/ml daptomycin. Statistically significant differences demonstrated by * indicating p<0.05 via a one-way ANOVA with Tukey-Kramer *post hoc* analysis. Data represents mean of N = 3 trials with error bars representing standard deviation.

Removing *sigM* or *glpF* did not significantly reduce bacterial susceptibility to daptomycin. High error bars mean that further trials should be conducted to ensure no significant differences were lost to experimental errors.

G. mellonella Virulence Assay



Worms were injected with either PBS, WT *B. anthracis* Sterne, **Δ**ClpX, **Δ**SigM, or **Δ**GlpF. Survival was plotted over 72 hours at 24-hour periods. 10 worms were utilized for **Δ**SigM, **Δ**GlpF, and PBS and 12 for WT and **Δ**ClpX. Statistically significant differences from wild-type demonstrated by * indicating P<0.05 via Log-rank test, Log-rank test for trend, and Gehan-Breslow Wilcoxon test. Data represents values from one trial.

While there was no statistically significant differences, there were noticeable differences in survival rates for *sigM* and *glpF* mutants compared to WT, and it is likely further trials would demonstrate significance.

Conclusions & Future Directions

Results indicated that *sigM* played a role in antibiotic resistance of *B. anthracis* Sterne. However, based off of the fact that ΔClpX reached its MIC before ΔSigM indicates that *sigM* is one of several genes, like *lrgAB*, that are in *clpX's* regulatory network affecting antibiotic resistance.

Future research should explore other genes like *msrA*. Given ClpXP proteases' potential as an antibiotic focus [7], better understanding how it is regulated could serve as a source for drugs targeting more antibiotic resistant bacteria.

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