ROLE OF SIGM, GLPF AND MSRA ON ANTIMICROBIAL SUSCEPTIBILITY AND VIRULENCE OF BACILLUS ANTHRACIS

by

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Master of Science, 2023

Texas Christian University

Fort Worth, Texas

Submitted to the Graduate faculty of the

College of Science and Engineering

Texas Christian University

In partial fulfilment of the requirements

For the degree of

Master of Science

March 2023

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Dissertation approved:

Major Professor

ACKNOWLEDGEMENTS

I want to express my gratitude to my research adviser, Dr. Shauna McGillivray, for her guidance and support. She has assisted me with experiment design, data analysis, data organization, and writing my paper. I want to acknowledge my former lab members, Graham Ellis and Vuong Do, for creating the insertional mutants necessary for my research. I want to thank TCU College of Science and Engineering for funding my research with a SERC grant. I would also like to thank Dr. Giridhar Akkaraju and Dr. Meredith Curtis from my committee for their constructive criticism of my research presentations. Finally, I would like to thank all my lab mates.

TABLE OF CONTENTS

LIST OF TABLES AND FIGURES

Introduction

Bacillus anthracis is a spore-forming, gram-positive bacterium with a rod shape (Thorne, 2014). It is the etiological agent of anthrax disease which usually affects herbivores and humans on rare occasions by exposure to anthrax spores from diseased animals and their products (Doganay & Demiraslan, 2015; Thorne, 2014). There are three types of anthrax in humans, cutaneous, pulmonary, and gastrointestinal anthrax, and their development depends on the route of infection (Dixon et al., 1999). Cutaneous anthrax occurs when pathogenic spores are introduced into our body through cuts or abrasion in the skin (Dixon et al., 1999). It is the most common form of anthrax that accounts for 95% of all human anthrax (Dognany & Welsby, 2006). Pulmonary or inhalational anthrax occurs through inhalation of *B. anthracis* spores (Dixon et al., 1999). Although this form of anthrax is rare, it can cause septic shock and is nearly always lethal (Calza et al., 2001). The third form of anthrax is gastrointestinal and oropharyngeal anthrax which is caused by the consumption of endospore-contaminated meat from infected animals (Dixon et al., 1999). This form of anthrax is also rare but can be fatal (Dixon et al., 1999).

Bacterial pathogens like *B. anthracis* produce virulence factors in order to replicate and spread throughout the host (Cross, 2008). The most well-studied virulence factors of *B. anthracis* are encoded on two plasmids, pXO1 and pXO2 (Spencer, 2003). The plasmid pXO1 encodes the genes responsible for toxin production (Bhatnagar & Batra, 2001). *B. anthracis* secretes two exotoxins: edema toxin and lethal toxin which suppress the host immune system. Both edema and lethal toxin include a component named protective antigen (PA) that binds to target cell receptors (Bhatnagar & Batra, 2001). The edema toxin is made up of PA and another protein called edema factor. The lethal toxin is made up of

PA and lethal factor (Bhatnagar & Batra, 2001). Receptor-mediated endocytosis is the process by which edema and lethal toxin are internalized after binding to the receptors. The endosome becomes acidified, which causes a pore to develop and allows the catalytic factor(s) to pass the endosomal membrane and enter the cytoplasm (Doganay & Demiraslan, 2015). The pXO2 plasmid carries the enzymes responsible for capsule production (Bhatnagar & Batra, 2001). Capsule is the outermost protective layer of a bacterium that protects the pathogenic bacteria from recognition and phagocytosis by host immune cells. They germinate inside macrophages and produce an antiphagocytic capsule, as well as the lethal and edema toxins, interfering with the host's defenses and causing destructive lesions (Bhatnagar & Batra, 2001; Spencer, 2003). The lymphatic system then spreads *B. anthracis* into the systemic circulation and other organs, resulting in sepsis (Bhatnagar & Batra, 2001; Dixon et al., 1999).

While anthrax toxin and capsule are both considered important virulence factors in *B. anthracis*, research suggests that chromosomal encoded genes may have a role in virulence as well (Read et al., 2003). To discover chromosomal genes involved in virulence, our lab previously created nearly 5000 mutants by transposon mutagenesis (McGillivray et al., 2009). The transposon can randomly insert into the chromosome of *B. anthracis* thereby disrupting the genes at or near the insertion point. These transposon mutants were then screened for loss of hemolytic activity as an indicator of bacterial virulence, which identified the *clpX* gene as involved in lysis of red blood cells (McGillivray et al., 2009). ClpX is a regulatory ATPase that can function independently as a chaperone or in combination with the caseinolytic peptidase (ClpP) to form the ClpXP protease (Baker and Sauer, 2011; Frees et al., 2007). ClpX identifies the damaged proteins,

unfolds the stable tertiary structure of proteins, and then translocates them into a proteolytic compartment of the enzyme, ClpP, where it is disassembled into smaller fragments (Baker and Sauer, 2011). ClpXP recognizes metabolic enzymes, stress response proteins, regulatory proteins, and damaged proteins, and it plays crucial roles in virulence, cell morphology, and initiation of developmental processes in gram positive bacteria (Baker & Sauer, 2012; Frees et al., 2007, 2014). The protease also regulates the activity and stability of key transcriptional regulators (Frees et al., 2007). ClpXP is therefore necessary for maintaining cell physiology and for global regulation of numerous genes (Frees et al., 2007; Michel et al., 2006). In *B. anthracis*, loss of ClpX leads to decreased virulence and increased susceptibility to antibiotics that target the cell envelope, either the cell wall or cell membrane, such as penicillin, daptomycin, and vancomycin (Claunch et al., 2018; McGillivray et al., 2009; Zou et al., 2021).

While *clpX* is critical for virulence in *B. anthracis*, it is unlikely to be directly mediating the effect. Rather genes in the regulatory network of *clpX* are likely directly contributing to virulence and/or antibiotic resistance in *B. anthracis*. Hence, our lab next investigated the genes that are differentially expressed (either up- or downregulated) in the Δ*clpX* mutant compared to the wild type *B. anthracis* through microarray analysis. This study found 119 genes that were highly differentially expressed in the Δ*clpX* mutant including *glpF, sigM, lrgA* and *lrgB,* which were downregulated in the Δ*clpX* mutant, and *msrA,* which was upregulated (Claunch et al., 2018). Our previous research focused on *lrgA* and *lrgB* and demonstrated they provide resistance to antibiotics that target the cell membrane like daptomycin and LL-37 in *B. anthracis* (Claunch et al., 2018). The role of the other three genes, *sigM, glpF* and *msrA* has not yet been investigated in *B. anthracis*

but all of these genes have been linked to antibiotic resistance in other bacterial species (Lu et al., 2003; Luo & Helmann, 2012; Utaida et al., 2003). Loss of *sigM* was linked with the increased susceptibility to cell-wall-targeting antibiotic called cefuroxime in *Bacillus subtilis* (Luo & Helmann, 2012). *glpF* is also involved in providing antibiotic resistance in *Staphylococcus aureus* as demonstrated by increased susceptibility to ampicillin or norfloxacin exposure in *glpF* mutants (Lu et al., 2003). The upregulation of *msrA* gene in *S. aureus* by the cell-wall-targeting antibiotics like bacitracin, oxacillin, and D-cycloserine implicate expression of *msrA* in resistance to these antibiotics (Utaida et al., 2003). Therefore, the major goal of my research is to see if *sigM*, *glpF* and *msrA* play a comparable role in *B. anthracis* and whether loss of these genes will lead to similar phenotypes as loss of *clpX*.

Methods

Table 1: Lists of the primers used.

Name of Primers	Primer Box #	Sequence
Up-F	8-31	TGT GTA CGG GCT CAA GAT TGC TTC
DN-R	8-35	CTA CGA CTG AAC ATA ACA ATC CAA CGC CA
CAT Fwd	3-96	ATG GAG AAA AAA ATC ACT GGA TAT ACC A
CAT Rev	3-97	TTA CGC CCC GCC CTG C
phYF	3-34	ACGACTCACTATAGGGCGAATTGG
phYR	3-35	CCGCTCTAGAACTAGTGGATCCCC

sigM-Rev	7-79	ACA GAA GCA CCC TCT TCG TAC
glpF-Rev	7-66	CGG ACC TAC TAC TGG AAT CCA TG
msrA-Rev	8-43	GCT ACA TTT GCT GGA GGA TGC
sigM-Up-Rev	8-33	CCC CCT CCT CCT TTC ACA TAT AAC ACG A

Bacterial strains and culture

This study used *Bacillus anthracis* Sterne (pXO1+, pXO2-) as the parental strain. *B. anthracis* Δ*clpX* mutant and the wild-type parental *B. anthracis* Sterne were previously described (McGillivray, Ebrahimi et al. 2009). Brain-Heart Infusion (BHI) media was used to culture WT *B. anthracis* at 37℃ under aerobic conditions. Chloramphenicol at 3 μg/ml was added to BHI to grow the Δ*clpX* mutant. The insertional mutants (IM) *sigM* IM, *glpF* IM, and *msrA* IM mutants were previously constructed (Ellis, 2020;Do, 2022). Briefly, these mutants were constructed by cloning approximately 350 bp of an internal fragment of the gene into the suicide plasmid pHY304 and selected using erythromycin at 5 μg/ml at 37℃ under aerobic conditions. The primer combinations phYF and sigM-Rev, phYF and glpF-Rev, phYF and msrA-Rev were used to confirm insertional mutation in *sigM* IM, *glpF* IM and *msrA* IM mutants respectively.

SigM knockout construct

The SigM targeting plasmid was constructed by a previous lab member (Geoff Hummelke). Briefly, 1000 bp upstream and downstream of the *sigM* gene were cloned into the pHY304 suicide plasmid using the technique described previously (Claunch et al., 2018). The targeting construct was sequenced using the primers phYF and sigM-Up-Rev to confirm the correct sequences were present and then transformed into electrocompetent GM2163, a methylation-deficient strain of *E. coli* and subsequently into *B. anthracis* using the technique described previously (Claunch et al., 2018). Before transforming into *B. anthracis,* the plasmid was isolated from GM2163 *E. coli* and purified. The success of transformation was confirmed by PCR using different primers and gel electrophoresis.

Generation of single and double cross-over events

To generate a single cross-over event, the positive clones of transformed *B. anthracis* were grown in BHI Erm5 overnight at 30°C. Approximately 25 µl of overnight cultures were transferred into 3 ml of fresh BHI Erm5 and grown for 8 hours at 37°C. Then, the 8-hour cultures were plated on BHI Erm5 agar plates and incubated overnight at 37°C. The colonies were then picked and screened for upstream and downstream cross over events. The primers Up-F and CAT Rev were used to confirm upstream single cross overs and CAT Fwd and DN-R were used to confirm downstream single cross overs.

To generate a double cross over event, colonies with an upstream cross over event were picked and grown overnight at 30°C shaking in BHI Cm3. 25 µl of overnight cultures

were inoculated into fresh BHI Cm3 and grown for 8 hours at 30°C shaking. The 8-hour culture was diluted 1:1000, plated on BHI Cm3 agar plates and incubated overnight at 37°C. The next day, the colonies were screened for a second crossover event by picking approximately 384 colonies into 96-well plates into either CM3 or Erm5 and monitoring for Erm sensitivity and CM resistance.

Minimum Inhibitory Concentration (MIC) assay

For the MIC assay with penicillin, overnight cultures of bacteria were grown in BHI at 37°C shaking. Then, 100 µl of overnight cultures were transferred to fresh BHI and grown to log phase at 37°C shaking until they reached optical density (OD) of 0.4-0.5 at a 600 nm wavelength. 10-fold serial dilution was performed and 1:10 dilutions were plated to calculate the colony forming units (CFU) per ml. The broth microdilution technique was used to conduct MIC assay. 100 µl of media with specified antibiotics in a series of two-fold diluted concentrations was transferred into 96-well plates. Then an equal volume of 1:10 dilution of bacterial culture was added. The plates were incubated at 37°C for 16-22 hours. Following that, the optical density was measured using a spectrophotometer. The identical procedure was followed for LL-37 and daptomycin except for an additional washing step. 1 ml of the log phase cultures were transferred into a centrifuge tube and centrifuged at 16,000 rpm for 5 minutes. The supernatant was then removed and replaced with 1 ml of PBS. It was centrifuged once more, the supernatant was removed and replaced with 1 ml of RPMI with 5% LB for MIC assay with LL-37 or replaced with MHB with 50 µg/ml of $CaCl_2$ for MIC assay with daptomycin. The final culture was diluted 1:10 with RPMI with 5% LB or MHB with 50 µg/ml of $CaCl_2$.

Acid stress assay

Overnight cultures of WT, *ΔclpX, sigM* IM, *glpF* IM and *msrA* IM mutants were grown in BHI at 37°C shaking. Then, 100 µl of overnight cultures were transferred to fresh BHI and grown to log phase at 37°C shaking until they reached optical density (OD) of 0.4-0.5 at a 600 nm wavelength. 1 mL of each log phase culture was transferred into centrifuge tubes, centrifuged at 16,000 rpm for 2 minutes, washed, and resuspended in an equal amount of PBS with a pH of 7 or PBS where the pH had been lowered to pH 4 with HCl. This process was repeated twice. Following a second wash, the cultures were diluted 1:2 with PBS (pH 7 or 4) and left on the bench at room temperature. The 1:2 dilution was diluted using 10-fold serial dilutions to enumerate colony counts every hour for a minimum of 4 to determine the approximate cfu/ml at each timepoint.

Growth curve

WT, *ΔclpX, sigM* IM, *glpF* IM and *msrA* IM mutants were cultured overnight in BHI at 37°C shaking. The overnight cultures were then transferred to fresh BHI and grown to log phase at 37°C shaking until they reached the optical density (OD) of 0.4–0.5 at a wavelength of 600 nm. 100 µl of log phase cultures were transferred into fresh BHI, which was then incubated at 37 °C shaking. After transferring log phase bacteria into fresh media, an OD reading was taken every hour for at least 8 hours, or until the bacteria reached stationary phase. The identical procedure was followed for RPMI with 5% LB except for the additional washing step. 1 ml of the cultures were transferred into a centrifuge tube after they reached the log phase and centrifuged them at 16,000 rpm for 5 minutes. The

supernatant was then removed and replaced with 1 ml of PBS. It was centrifuged once more, the supernatant was removed and replaced with 1 ml of RPMI with 5% LB. The final culture was diluted 1:10 with RPMI with 5% LB.

Results

Construction of *sigM* knockout and insertional mutants and their confirmation

Since *sigM*, *glpF* and *msrA* have all been linked to resistance of cell-wall targeting antibiotics in other gram-positive bacterial species and since they are all part of the ClpX regulon, I hypothesized that these might also confer resistance to cell-wall targeting antibiotics in *B. anthracis*. To test this hypothesis, I first attempted to construct a genetic knockout of *sigM*. This gene seemed a particularly promising target as it encodes the RNA polymerase sigma factor SigM. Sigma factors regulate transcription in bacteria and play a major role in transcription initiation (Davis et al., 2017). SigM specifically has been found to confer resistance to cell-wall targeting antibiotics and cell wall integrity in the closely related gram-positive bacterium, *Bacillus subtilis* (Luo & Helmann, 2012).

Geoff Hummelke, a former lab member, created a temperature-sensitive *sigM* targeting construct in the pHY304 plasmid. It contained *cat* (chloramphenicol acetyltransferase), a chloramphenicol resistance gene, in the center, along with the 1000 bp upstream (UP) and downstream (DN) regions of *sigM*. As seen in figure 1A, it also contained the erythromycin resistance gene (Erm).

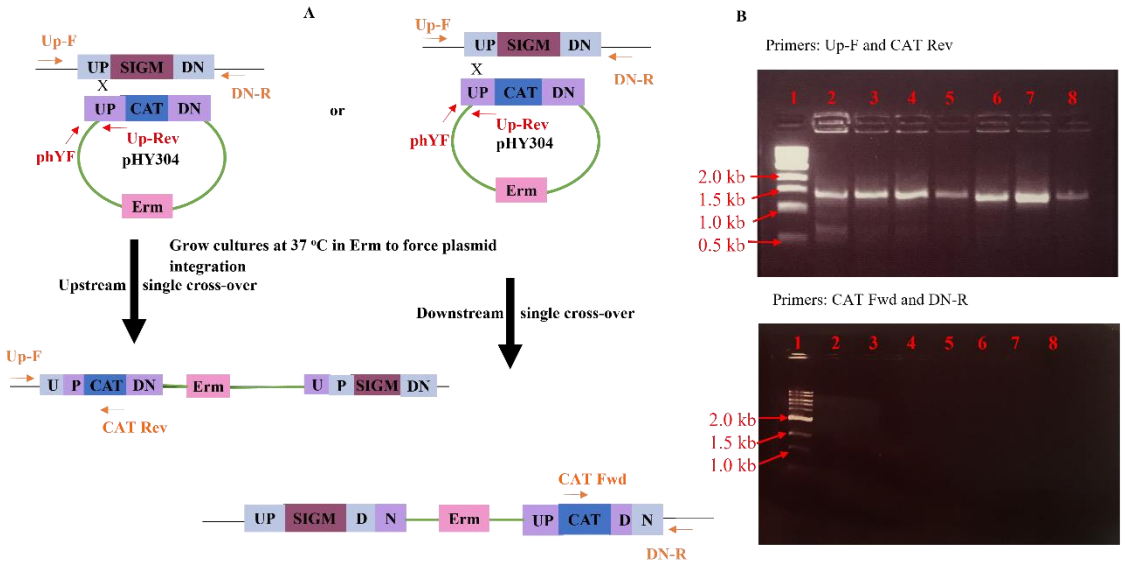

Figure 1: Schematic diagram showing the generation of single cross-over events. (A) Schematic diagram for the generation of single cross-over event (B) Top panel: Colony PCR to screen the upstream cross-over event using Up-Fwd and Cat Rev primers. C) Bottom panel: Colony PCR to screen downstream cross-over event using Cat Fwd and DN-Rev primers (CAT: Chloramphenicol resistance gene, Erm: Erythromycin resistance gene, UP: 1000 bp upstream of *sigM,* DN: 1000 bp downstream of *sigM)* (1=DNA Ladder). Primers are indicated by arrows in orange and red, genomic DNA is black, plasmid DNA is green, and X represents crossing over.

I confirmed that the plasmid contained the upstream regions of *sigM* gene by PCR using pHYF and Up-Rev primers (red primers located on the schematic diagram in figure 1A) and then by gene sequencing. The gene sequencing verified that the knockout plasmid contained upstream and downstream regions of *sigM* and the *cat* gene. The plasmid was then transformed into electrocompetent GM2163, a methylation-deficient strain of *E. coli* and subsequently into electrocompetent *B. anthracis.* The success of transformation into *B. anthracis* was confirmed by screening approximately 6 colonies using CAT Fwd and CAT Rev primers and gel electrophoresis.

To generate a single crossover event, the transformed colonies were grown at 37℃ under erythromycin selection. The plasmid has a temperature-sensitive origin of replication, so it cannot replicate at 37℃, forcing plasmid integration into the bacterial genome. The plasmid integration occurs via homologous recombination into either the upstream or downstream regions of the *sigM* gene resulting in an upstream crossover or downstream crossover respectively as indicated in figure 1A. I screened approximately 28 colonies using the primers Up-F, a genomic primer upstream of the plasmid insertion site and CAT Rev, plasmid-specific primer for an upstream crossover event. This would generate an approximately 1200 bp band in the case of an upstream single-cross over and no band with a downstream single cross-over (see figure 1A for primer locations marked by orange arrows). Similarly, I used CAT Fwd, plasmid-specific primer and DN-R, a genomic primer downstream of the plasmid insertion site for a downstream crossover event as this would only generate a band in the case of recombination at the downstream region of homology. All 28 colonies generated an upstream crossover event, the results for 7 colonies are shown in figure 1B (upper panel). There were no downstream crossover events (figure 1B lower panel). This indicates that there is a strong preference for homologous recombination to occur at the upstream region of the genome rather than the downstream region.

Even though we had no colonies with the downstream crossover, we attempted to proceed with only the upstream single crossover colonies to produce a double crossover event. To generate Δ*sigM*, the second recombination event must occur at the downstream region. This will cause the *cat* gene to remain in the genome, but the *sigM* gene to be

removed on the re-formed suicide plasmid, which will eventually be lost when grown at 37°C (Figure 2).

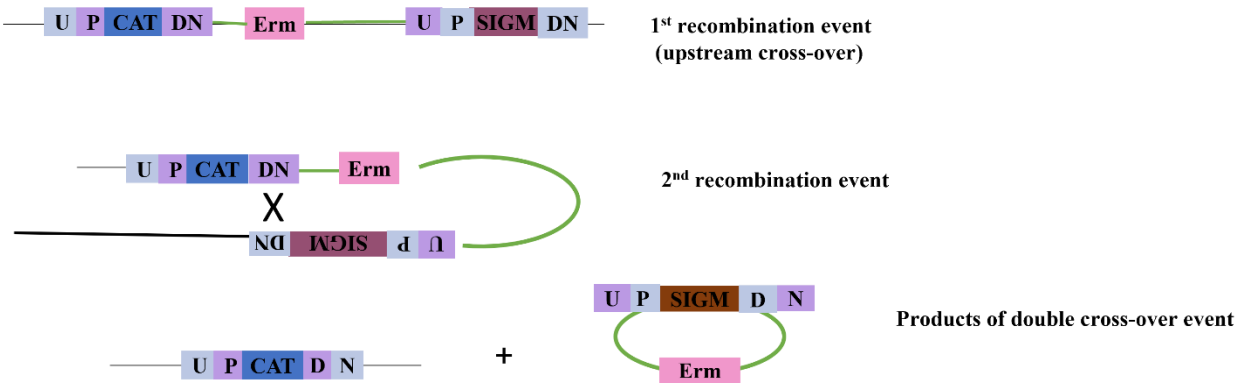

Figure 2: Schematic diagram showing the generation of double cross-over events. Genomic DNA is black, plasmid DNA is green, X represents crossing over.

Unfortunately, our results with the single-cross overs had already demonstrated that recombination at the downstream region was not favorable. Despite these odds, we screened colonies for a second crossover event by picking approximately 384 colonies into 96 well plates containing either CM3 or Erm5 and growing overnight. Colonies with a single cross-over will have both CM3 and Erm5 resistance, however, colonies where a successful second recombination event occurred will lose the plasmid over time and become sensitive to Erm5. The next day we monitored for colonies that had Erm sensitivity and Cm resistance, but unfortunately every colony had Cm3 and Erm5 resistance. Therefore, we concluded it would not be possible to generate a double crossover event using this targeting construct.

Since our efforts at creating a true genetic knockout using a double crossover failed, we instead turned to insertional mutagenesis, which relies only on a single recombination event where the targeting plasmid is inserted into the middle of the target gene making the gene non-functional. While technically easier there are downsides to insertional mutagenesis. They are not as stable as a true knockout, it is possible for the plasmid to pop back out of the genome resulting in a wildtype reversion and, because the entire plasmid is inserted in the genome, it can cause dysregulation of any downstream genes in the operon. Although the plasmid inserted in the middle of the target gene brings its own promoter (blue arrow in figure 3), it is not the native promoter and regulation of genes downstream of the inserted region will be affected if they are on the same operon. The *sigM* and anti-*sigM* genes are regulated as an operon (figure 3).

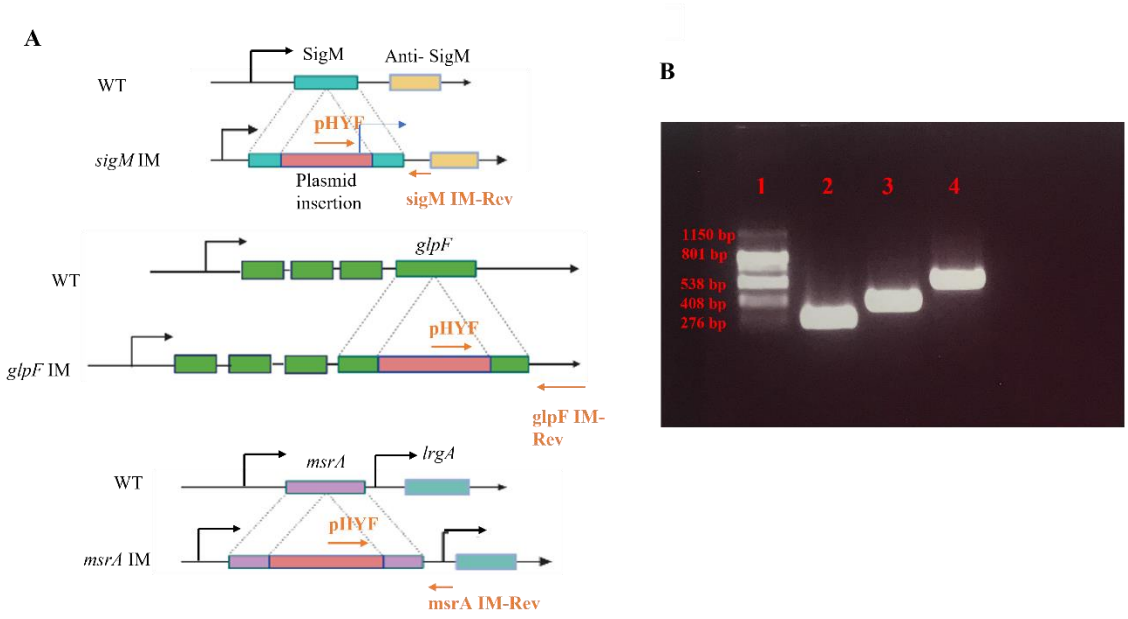

Figure 3: Construction and confirmation of insertional mutants
(A) Schematic diagram and (B) PCR confirmation of insertional mutation in *sigM* IM, *glpF* IM and *msrA* IM mutants with Lanes 1: DNA ladder, 2: *sigM* IM 3: *glpF* IM and 4: *msrA* IM mutant. Primers are indicated by arrows in orange. The arrows in black and blue represent the promoter. The black promoter comes from the genome and the blue promoter comes from the plasmid.

However, since we could not generate the true knockout of *sigM* we instead used the insertional mutant of *sigM* that had already been created by an earlier lab member, Graham Ellis (Ellis, 2020). Insertional mutation is less of an issue for *glpF* and *msrA* since *glpF* is the last gene in the operon (figure 3) and does not affect the regulation of other downstream genes. Similarly, the gene downstream of *msrA* is controlled by a different promoter (figure 3), therefore an insertional mutation in *msrA* should not alter its expression. Therefore, we used *glpF* and *msrA* insertional mutants created by previous lab members, Graham Ellis and Vuong Do respectively (Ellis, 2020; Do, 2022). Before running any assays, I confirmed these insertional mutants. *sigM* IM, *glpF* IM and *msrA* IM were verified using the forward and reverse primers as indicated in figure 3. Among

the primers used to confirm insertional mutants, pHYF is a plasmid-specific promoter while *sigM* IM-Rev, *glpF* IM-Rev and *msrA* IM-Rev are the genomic primers located downstream of the plasmid insertion site. These primer combinations can only amplify DNA when the plasmid has been correctly inserted into the gene, which is then verified by gel electrophoresis. The disruption of the *sigM*, *glpF*, and *msrA* genes as well as the plasmid insertion were confirmed by the observation of DNA band in the *sigM* IM, *glpF* IM, and *msrA* IM mutants in gel electrophoresis, as shown in Figure 3B. The expected band size for *sigM* IM, *glpF* IM, and *msrA* IM mutants were approximately 392 bp, 550 bp and 745 bp respectively.

Loss of *sigM, msrA and glpF* increases susceptibility to penicillin but not daptomycin.

We predicted that the loss of *sigM* and *glpF* would have comparable effects to the loss of *clpX* in *B. anthracis* since *sigM* and *glpF* are both downregulated in the Δ*clpX* mutant. At 20 µg/ml of penicillin, a significant difference between the growth of WT and the *glpF* IM mutant was observed in stationary phase whereas the growth of the WT and the *sigM* IM mutant differed significantly at 20 µg/ml of penicillin in log phase. The loss of *sigM* and *glpF* had less of an effect on penicillin resistance as the loss of *clpX*, with inhibition of growth only observed at concentrations of 20 µg/ml or greater in both the *sigM* and *glpF* mutants. Therefore, we concluded that *glpF* and *sigM* are required for *B. anthracis* to provide penicillin resistance, although in a growth phase-dependent manner.

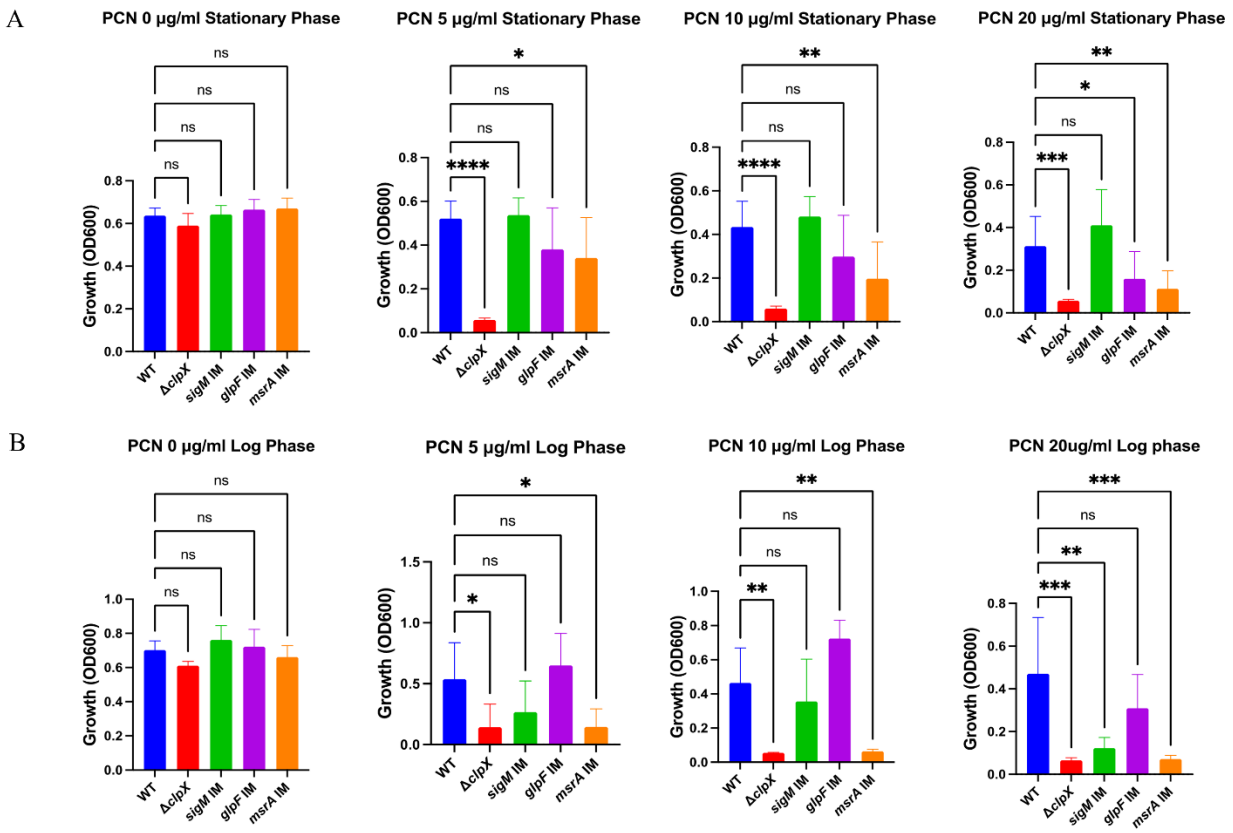

Figure 4: *sigM*, *msrA* and *glpF* are necessary in *B. anthracis* to provide resistance against penicillin.
Growth of WT, Δ*clpX*, *sigM* IM, *glpF* IM and *msrA* IM mutants at varying concentrations of penicillin (PCN) in (A) stationary phase (B) log phase. Data is represented as mean +/- SD. At least three independent experiments were conducted. * indicates a p-value less than 0.05, ** represents a p-value less than 0.01, *** represents a p-value less than 0.001, **** represents a p-value less than 0.0001, as determined by one-way ANOVA. ns indicates non-significant difference. ns indicates non-significant difference.

As *msrA* is upregulated in Δ*clpX* mutants, we predicted an opposite phenotype in *msrA* IM mutants with respect to Δ*clpX*. We reasoned that because the Δ*clpX* mutant had increased susceptibility to penicillin and increased *msrA* expression, then loss of *msrA* expression might lead to increased penicillin resistance. Nevertheless, when tested for antibiotic susceptibility using penicillin, the *msrA* IM exhibited a similar phenotype to the Δ*clpX* mutant. Even at a low concentration of penicillin, 5 µg/ml, *msrA* IM mutant was

susceptible to penicillin both in stationary phase and log phase with sensitivity comparable to Δ*clpX* mutant in log phase. Consequently, we concluded that *msrA* is crucial for *B. anthracis* to provide penicillin resistance and, at least when using log phase cultures, is nearly as sensitive as the Δ*clpX* mutant.

The antimicrobial susceptibility of *sigM* IM, *glpF* IM and *msrA* IM mutants to daptomycin, an antibiotic that targets cell membranes, was next examined. Daptomycin binds to the cell membrane of gram-positive microorganisms and quickly depolarizes the membrane potential, which inhibits the synthesis of DNA, RNA, and proteins inside the cell. Bacterial cell death eventually follows from this inhibition (Patel & Saw, 2023). We anticipated that the loss of *sigM* and *glpF* would have effects like Δ*clpX* in *B. anthracis*. However, we only noticed a very slight difference between WT and *sigM* IM mutant at 4 µg/ml of daptomycin in log phase, and we did not notice any difference between *glpF* IM and WT, or *msrA* IM and WT (figure 5). As a result, we concluded that neither *glpF* nor *msrA* are important for daptomycin resistance in *B. anthracis*, and the role of *sigM* is negligible.

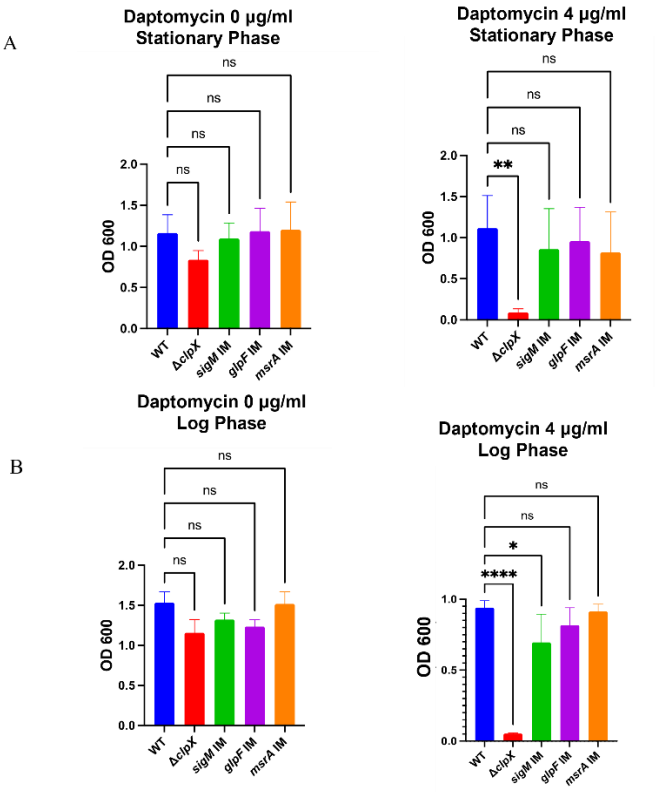

Figure 5: *sigM, msrA* and *glpF* are not required in *B. anthracis* to provide resistance against daptomycin.
Growth of WT, *ΔclpX*, *sigM* IM, *glp*F IM and *msrA* IM mutants at 0 µg/ml and 4 µg/ml of daptomycin using cultures in(A) stationary phase (B) log phase. Data is represented as mean +/- SD. At least three independent experiments were conducted.
* indicates a p-value less than 0.05, ** represents a p-value less than 0.01, **** represents a p-value less than 0.0001, as determined by one-way ANOVA. ns indicates non-significant difference. ns indicates non-significant difference.

Loss of *sigM, glpF* and *msrA* do not show susceptibility to heat stress while loss of *glpF* increases susceptibility to acidic stress.

Previous studies conducted by our former lab member, Vuong Do demonstrated the necessity of *clpX* for growth of *B. anthracis* under heat stress (Do, 2022). Thus, I hypothesized that the genes that are regulated by *clpX* might also be involved in heat stress tolerance. To test this hypothesis, the growth of wild type (WT), *clpX*, *sigM* IM, *glpF* IM, and *msrA* IM mutants was first evaluated at 37°C as a control to determine if the loss of *clpX*, *sigM*, *glpF*, and *msrA* genes affect the growth of *B. anthracis* under physiological temperature. By comparing the growth curves of WT, Δ*clpX*, *sigM* IM, *glpF* IM, and *msrA* IM mutants in BHI at 37 °C (figure 6 left upper panel), we found that all the mutants grew at the same rate as compared to WT.

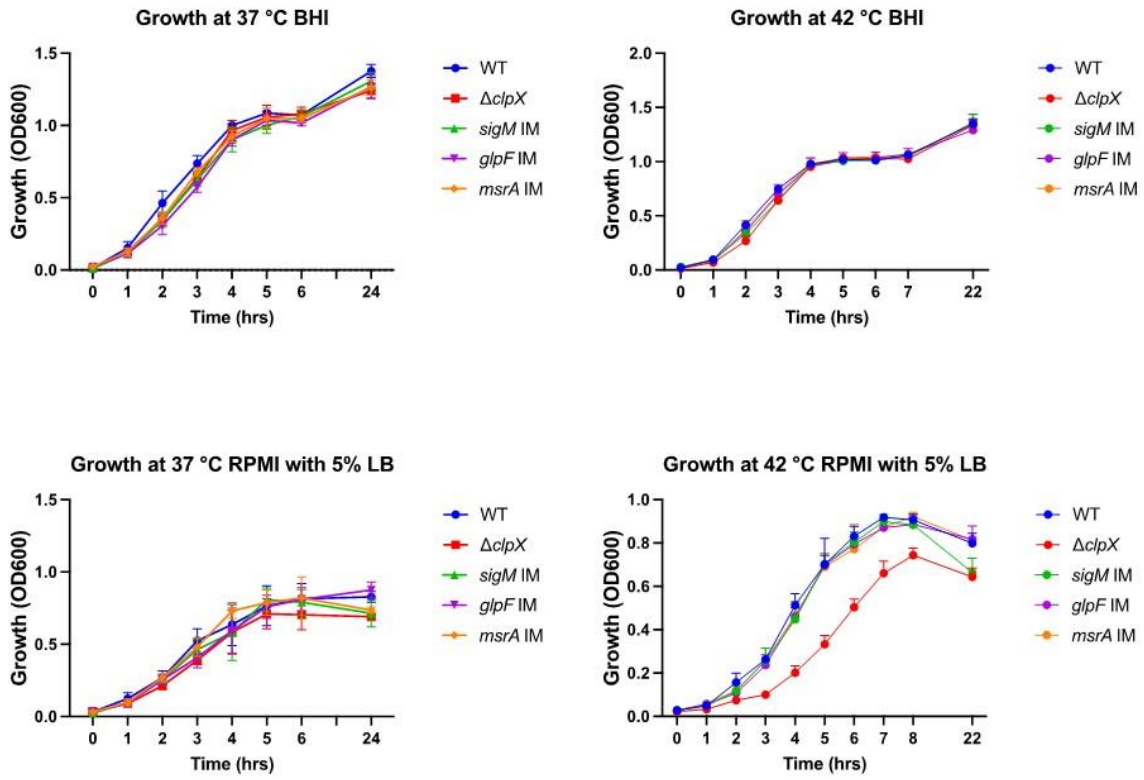

Figure 6: *sigM, glpF* and *msrA* are not critical in providing tolerance to heat stress at 42℃. Growth of wild-type *B. anthracis*, *ΔclpX, sigM* IM*, glpF* IM and *msrA* IM mutants in BHI and RPMI with 5% LB at physiological temperature (37℃) and at heat stress (42℃). Data is presented as mean +/- SD of three independent experiments.

Therefore, we concluded that the loss of the *clpX*, *sigM*, *glpF*, and *msrA* genes do not impact the growth of *B. anthracis* in BHI. The test was also carried out in RPMI containing 5% LB. BHI is an ideal medium for growing microorganisms, whereas RPMI is a medium for growing mammalian cells and may better represent the conditions found in the host. The growth curve in RPMI at 37°C was done to see if the change in culture medium would affect the growth of *B. anthracis* under physiological temperature, but we discovered all the mutants grew at the same rate as WT (figure 6 right upper panel). After it was discovered that the loss of the *clpX, sigM, glpF,* and *msrA* genes had no impact on the growth of *B. anthracis* at 37°C in both BHI and RPMI, the heat stress test was performed

at 42°C. From this test, it was determined that all the mutants could grow at the same rate as WT in BHI at 42°C (figure 6 left lower panel), but the growth of Δ*clpX* mutant in RPMI with 5% LB medium was slightly slower than that of WT (figure 6 right lower panel). As a result, we concluded that heat stress does alter growth for the Δ*clpX* mutant but only in the less nutrient rich RPMI medium.

Previous studies conducted by our former lab member, Vuong Do also demonstrated the necessity of *clpX* for acid stress tolerance in *B. anthracis* (Do, 2022). Thus, I hypothesized that the genes that are regulated by *clpX* might also be involved in acidic stress. To determine if the genes are involved in acidic stress tolerance, an acid stress test was performed. As expected, the survival of WT, Δ*clpX*, *sigM* IM, *glpF* IM, and *msrA* IM was not affected at pH 7. We observed the bacterial population density until 4 hours and found that the population density of the mutants was similar to WT at pH 7 (figure 7 left).

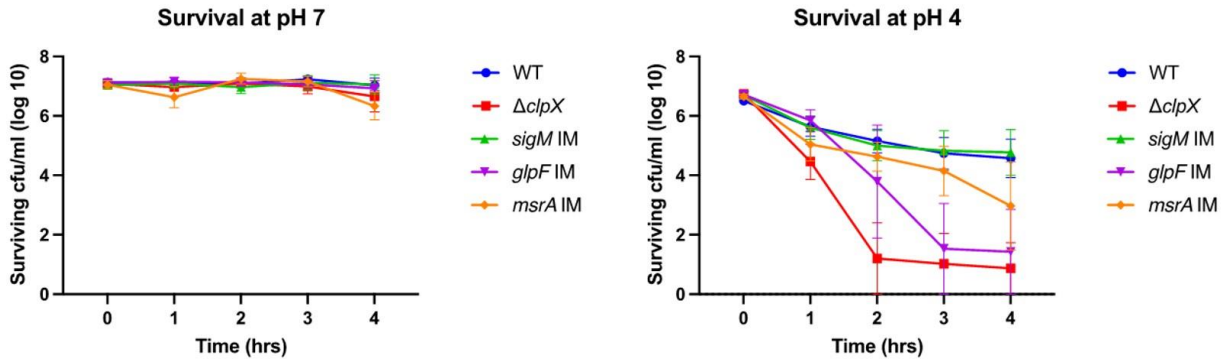

Figure 7: Loss of *glpF* in *B. anthracis* increases bacterial susceptibility to an acidic environment. Survival assay of the wild-type *B. anthracis, ΔclpX, sigM* IM, *glpF* IM and *msrA* IM mutants at pH7 or pH4 at 1 hour interval until 4 hours. Data is represented as mean +/- SD. At least three independent experiments were conducted.

We performed the same experiment at pH 4 and found that the surviving bacterial population of *glpF* IM mutant was lower than that of WT, which was comparable to that of the Δ*clpX* mutant (figure 7 right). We therefore concluded that *glpF*, but not *msrA* or *sigM*, is required for *B. anthracis* Sterne to survive in an acidic pH.

Discussion

Earlier studies discovered that the loss of the ClpX or ClpXP protease in *B. anthracis* causes a decrease in virulence and an increase in susceptibility to antibiotics such as penicillin, daptomycin, and vancomycin (McGillivray et al., 2009; Zou et al., 2021). Deletion of ClpX in *B. anthracis* results in deregulation of numerous genes, including *sigM*, *glpF*, *msrA*, *lrgA*, and *lrgB*, all of which are crucial for providing antimicrobial resistance in closely related species. The *lrgA* and *lrgB* genes have already been investigated by Kevin M. Claunch, a former lab member, and loss of these genes were found to have similar although not identical phenotypes as loss of *clpX* (Claunch et al., 2018). Therefore, we suspected other genes might contribute to the phenotypes associated with loss of *clpX*. My objective was to investigate the *sigM*, *glpF*, and *msrA* genes to determine their role in antibiotic resistance and other ClpX-associated phenotypes.

sigM, which encodes the RNA polymerase sigma factor SigM, has been found to confer resistance to cell-wall targeting antibiotics in another gram-positive bacterium, *Bacillus subtilis* (Luo & Helmann, 2012). Sigma factors are important regulators of transcription in bacteria and are involved in initiating RNA synthesis. (Davis et al., 2017). Each sigma factor can potentially regulate large sets of genes and some sigma factors like

SigB are induced in response to specific environmental stress signals in *B. subtilis* and related gram positive bacteria (Hecker et al., 2007). Loss of SigM was linked with the increased susceptibility to cell-wall-targeting antibiotic called cefuroxime in *Bacillus subtilis* (Luo & Helmann, 2012). SigM has also been found to be crucial for regulation of bacterial genes responsible for maintaining cell-wall integrity (Luo & Helmann, 2012). Despite the literature-based evidence of the role of *sigM* in maintaining cell wall integrity and antibiotic resistance, the growth of *B. anthracis* was only modestly affected in our study in the presence of penicillin, a cell-wall-targeting antibiotic. Significant growth inhibition was only observed at the highest penicillin concentration (20 µg/ml) compared to the wild-type *B. anthracis* Sterne. SigM was also found to be activated in response to different stresses like ethanol and acid (Thackray & Moir, 2003). Mutants of SigM were unable to grow at low pH (4.3) (Thackray & Moir, 2003). However, when we tested the survival of *B. anthracis* under acidic stress at pH 4, we saw no change in survival from the wild-type strain even at 4 hours of treatment with acidic stress at pH 4, showing that *sigM* is not required to provide acidic stress tolerance in *B. anthracis*. These modest phenotypes were somewhat unexpected given the known function of *sigM* in regulating cell wall homeostasis and response to stress. It is possible that the unexpected result may be due to the insertion of a plasmid in the *sigM* gene, which could have caused dysregulation of the anti-*sigM* gene that is located next to *sigM* and is controlled by the same promoter. Anti-*sigM* negatively regulates the expression of the *sigM* gene in *B. anthracis*, and overexpression of anti-*sigM* would decrease the ability of the bacterium to survive under stress conditions. To confirm whether the anti-*sigM* gene is dysregulated in the *sigM* IM mutant, mRNA expression analysis can be conducted. This would help determine whether

the observed phenotype is due to dysregulation of anti-*sigM* or other factors and provide a better understanding of the role of the *sigM* in the stress response of *B. anthracis*. Future experiments will also attempt to knockout anti-*sigM* alone and in combination with *sigM* to determine the relative roles of each of these genes.

There is less known about *glpF* and *msrA* and their connection to antibiotic resistance. *glpF* encodes a cytoplasmic membrane protein called glycerol uptake facilitator protein that facilitates the uptake and metabolism of glycerol, G3P, and glycerophosphorylphosphodiesters in a cell (Lu et al., 2003; Sweet et al., 1990; Weissenborn et al., 1992). *glpF* is also involved in providing antibiotic resistance in *Staphylococcus aureus* as demonstrated by an increase in susceptibility to ampicillin or norfloxacin exposure in *glpF* mutants (Han et al., 2014). Our findings indicate that *glpF* is required for stationary phase *B. anthracis* to develop resistance to the antibiotic penicillin as our mutant showed a modest growth defect upon exposure to the antibiotic. The role of *glpF* in stress tolerance has not been well studied in bacteria. A few studies investigated the role of *glpF* in the fungus *Neurospora crassa* and discovered the importance of *glpF* to withstand high salt concentrations (Liu et al., 2015). It has also been linked with high osmosis tolerance under stressor-treatment settings in yeast (Liu et al., 2015). Our results from the acidic stress test at pH 4 show that *glpF* plays a crucial role in *B. anthracis* to withstand acidic stress. As mentioned earlier, *glpF* encodes a membrane protein that facilitates the transport of glycerol across the cell membrane. Glycerol is a crucial source of energy for the cell, and the maintenance of glycerol uptake and metabolism is essential for the survival of bacteria under various stress conditions, including acidic stress. According to Guan and Liu (2020), the effects of acidic environments can be counteracted

by maintaining the balance of pH within the cell, protecting the integrity and flexibility of the cell membrane, and regulating metabolism (Guan & Liu, 2020). It is possible that *glpF* plays a critical role in metabolism under acidic stress conditions, thereby helping to maintain pH homeostasis of the cell. Further studies are needed to elucidate the exact mechanisms by which *glpF* regulates metabolism under acidic stress in *B. anthracis*.

msrA encodes an enzyme called methionine sulfoxide reductase that reduces protein-bound methionine sulfoxide (Met(O)) and restores its function and therefore plays an important role in antioxidation (Achilli et al., 2015; Duport et al., 2021). The role of *msrA* in oxidative stress tolerance has been extensively studied, but little is known about its function in antibiotic resistance. In our study, the loss of *msrA* increased the susceptibility to penicillin and the bacterial growth was comparable to that in *ΔclpX* mutant demonstrating that expression of *msrA* is required in providing resistance to penicillin in *B. anthracis.* This is surprising since the *msrA* gene is upregulated in Δ*clpX* mutant and we expected an opposite phenotype with respect to Δ*clpX* mutant. One possible explanation could be that the upregulation of *msrA* in the absence of *clpX* may be a compensatory mechanism to counteract the loss of *clpX*. Nevertheless, our results do fit with those seen in *S. aureus* where an increased in *msrA* expression is linked with resistance to the cell-wall-targeting antibiotics like bacitracin, oxacillin, and D-cycloserine (Utaida et al., 2003). Therefore, the Utaida et al study supports our data showing that a loss of *msrA* increases resistance to cell-wall active antibiotics, which fits with our penicillin data.

Next, we investigated whether *sigM*, *glpF*, and *msrA* are required to provide resistance to heat stress at 42℃. According to our findings, none of these genes are required for providing resistance to heat stress at 42℃. We did notice that the Δ*clpX* mutant grew

slightly slower than the WT at 42℃ in RPMI, but not in BHI. Based on this observation, we predicted that BHI may contain nutrients or other factors that support the growth of Δ*clpX B. anthracis* even in adverse conditions, while RPMI may lack these factors and therefore make it more difficult for the bacteria to grow under stress.

In conclusion, we discovered that the loss of each of the three genes we studied resulted in increased susceptibility to penicillin, although only *msrA* IM had a similar level of growth inhibition as loss of *clpX*. Interestingly, our mutants did not show a significant impact in daptomycin resistance despite a strong phenotype in Δ*clpX*. Future experiments will look at the role of other cell-wall targeting antibiotics such as vancomycin and the antimicrobial peptide LL-37. In regard to other ClpX-related stress responses, our study found that only *glpF* conferred resistance to acidic stress, and the phenotype was strong and comparable to Δ*clpX* mutant. None of our mutants were found to be necessary for providing heat stress resistance. Taken together, these data indicate that the phenotypes seen in the Δ*clpX* mutant are likely multi-factorial, with different downstream genes playing different roles and that loss of no one gene will result in the same phenotype as loss of *clpX*.

Overall, this study has improved our understanding of the regulatory network of ClpX in antibiotic resistance and stress response in *B. anthracis*. By identifying the specific genes and pathways involved in virulence and antibiotic resistance in *B. anthracis*, this study may help in the identification of new targets for antibiotic therapy and the development of more effective treatments for anthrax infections. Furthermore, the insights gained from this study may be applicable to other bacterial pathogens and may help in the development of treatments for other antibiotic-resistant infections.

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ABSTRACT

ROLE OF SIGM, GLPF AND MSRA ON ANTIMICROBIAL SUSCEPTIBILITY AND VIRULENCE OF BACILLUS ANTHRACIS

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Bacillus anthracis is a gram-positive bacterium that causes the deadly anthrax disease. ClpX is a subunit of ClpXP protease that is known to be essential in virulence as well as providing resistance to cell-envelope targeting antibiotics such as penicillin, daptomycin, and the antimicrobial peptide LL-37. While *clpX* is critical for virulence in *B. anthracis*, it is unlikely to be directly mediating the effect. Hence, our lab investigated the genes that are differentially expressed in the Δ*clpX* mutant compared to the wild type *B. anthracis* through microarray analysis. We found 119 genes that were highly differentially expressed in the Δ*clpX* mutant. In this study, we focused on three genes *sigM* and *glpF*, which are downregulated in the Δ*clpX* mutant, and *msrA*, which is upregulated in the Δ*clpX* mutant because *sigM* confer resistance to cell-wall targeting antibiotics in the closely related gram-positive bacterial species, *Bacillus subtilis* while both *glpF* and *msrA* confer resistance in *Staphylococcus aureus*. Our objective was to determine whether loss of *siM, glpF* and *msrA* will lead to similar phenotypes as loss of *clpX* in *B. anthracis* Sterne. We found that *sigM*

and *glpF* are more susceptible to penicillin, although in a growth phase dependent manner, and only *glpF* is critical for daptomycin resistance. Loss of *msrA* increased the susceptibility to penicillin in both log and stationary phase which was comparable to loss of *clpX*. Our findings from the heat stress test at 42 °C indicate that the *sigM*, *glpF*, or *msrA* genes might not be required to confer heat stress resistance in *B. anthracis* since all these mutants grew at the same rate as WT. The loss of *glpF* reduced the number of surviving cfu/ml in the acidic stress test at pH 4, which was comparable to the Δ*clpX* mutant, indicating that *glpF* is crucial for *B. anthracis* to provide acidic stress resistance. Future studies will examine the susceptibility of these mutants to LL-37 and vancomycin. Complementation of these mutants will serve to further support the importance of these genes for the roles we examined. This research will aid in understanding the mechanism of antibiotic resistance and virulence in the ClpX regulatory network in *B. anthracis*.