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Investigating the Role of Two ATP-Dependent Proteases, *ftsH* and *clpC*, in Nitric Oxide Resistance Present in *Staphylococcus aureus*

Aurora Eichmiller
Department of Biology
The University of North Carolina Asheville
One University Heights
Asheville, North Carolina

Faculty Research Advisor: Dr. Melinda Grosser

Abstract

Staphylococcus aureus is a highly successful pathogen due to its wide range of antibiotic and immune resistance mechanisms. One unique and concerning resistance factor involves *S. aureus* resistance to the antimicrobial radical nitric oxide (NO·). NO· is a membrane permeable gas produced by activated phagocytes to function as an antimicrobial agent against pathogens. NO· is usually an effective defense against bacteria; however, *S. aureus* is unique in that it is still able to replicate under nitrosative stress. The capability to persist under high levels of NO· is complex and hinges on the use of various genes and metabolic enzymes. *FtsH* is an ATP-dependent, zinc-binding metalloprotease located in the cell membrane of *S. aureus* and is critical for survival under environmental stressors. *FtsH*, as well as other proteolysis enzymes, play important roles in the elimination of misfolded and non-functional proteins. Although it has been implicated in NO· resistance with the use of deep sequencing of transposon junctions, its specific role during NO· stress is unknown. *FtsH* modulates levels of proteins potentially involved in both heme synthesis and respiration such as *cydA*, *cyoE*, and *hemeA*. NO· is capable of damaging heme and respiratory enzymes by reacting with iron, which temporarily inhibits respiration in *S. aureus*, so we hypothesized that *ftsH* plays a role in regulating damage to the electron transport chain. Here, we utilize a modified allelic exchange method to create an *ftsH* deletion mutant. Future studies will define the role of *ftsH* during NO·, specifically how it impacts recovery of respiration

1. Introduction

S. aureus's success as an invasive pathogen is demonstrated through its ability to rapidly evolve antibiotic resistance. While it is known to asymptomatically colonize one-third of the healthy human population, it is also a leading cause of skin and soft tissue infections (SSTI's) [1] . The persistence of Methicillin Resistant *S. aureus* (MRSA) throughout the world has reinforced the urgency we face in the research and development of novel antimicrobial therapeutics. The development of new antibiotics hinges on the ability to identify the existing mechanisms that S. aureus utilizes to not only persist after medical intervention with antimicrobials, but also to evade host innate immune defenses. A first line defense against pathogens, the innate immune system consists of three effector mechanisms, including enzymes and antimicrobial peptides, the complement system, and phagocytes [2]. One particular antimicrobial agent employed by the innate immune system, nitric oxide (NO·), is produced by phagocytes and is typically very effective at combating bacterial infection within the host. However, S. aureus is unique among bacterial pathogens in the fact that it is able to both resist and continue growing in the presence of NO· [3].

NO· is a radical membrane-permeable gas that plays a vital role in the phagocyte-mediated response, and is used to fight against fungi, bacteria, and viruses within the infected host [3]. NO· is synthesized from the amino acid L-arginine by a family of enzymes called nitric oxide synthases (NOS), and does so through the mechanism of a specific metabolic route deemed the L-arginine-NO pathway [4]. The mechanisms by which NO· deters pathogens and subsequent infection

is twofold; in one way acting as a signaling molecule to promote the growth and action of immune cells, and through the covalent binding of bacterial DNA, proteins, and lipids. Due to its small size and its lipophilic and hydrophilic properties, NO· is able to easily permeate the membrane of bacterial cells and cause nitrosative damage by altering cell DNA, inhibiting enzyme function through interaction with heme groups and iron sulfur clusters, and by inducing the peroxidation of lipids [5]. Proteins that contain heme are particularly efficient targets for NO·, as it is able to irreversibly bind to heme, which can result in the removal of heme from the target bacterial metalloenzyme. The removal of heme will eventually cause severe iron depletion for the bacterial cell, and effect the cell's ability to respire, replicate and grow [5].

These consequences described above are typical for most bacterial species that attempt to infect their host; however, *S. aureus* has evolved mechanisms to avoid the costs associated with exposure to NO·. One way that *S. aureus* overcomes the threat posed by NO· is through the utilization of a unique two-component signaling system called the staphylococcal respiratory response AB (SrrAB) to both sense its environment and to adapt to stressors used by its host. This system enables *S. aureus* to survive and persist under nitrosative stress by connecting environmental stimuli to the needed cellular response through the sensor histidine kinase and an effector response regulator [6]. Metabolic adaption to nitrosative stress by *S. aureus* is also enabled through the NO· inducible L-lactate dehydrogenase (ldh1), an allele not found in any other Staphylococcal species. Ldh1 is transcribed from the NO· detoxifying flavohemoglobin (Hmp), functioning to catalyze the reduction of pyruvate to L-lactate with the accompanying oxidation of NADH to NAD^+. This process serves the purpose of restoring redox balance within the cell when it is has lost the ability to continue aerobic respiration due to the buildup of NO· [7]. In addition to these regulatory genes mentioned above, further regulators that have been characterized in mitigating the effects of NO· include *SarA, CodY, Rot,* and *fur*. Through the construction of deletion mutants, these regulatory proteins were found to be highly sensitive to exposure of NO· and displayed disruption of metabolic processes and transcriptional regulation when under nitrosative stress [3]. Although we have an understanding of several NO· response factors, identification and knowledge regarding the regulatory mechanisms of these response factors is lacking, thus making further investigation of these mechanisms essential.

In an effort to identify other genes that contribute to NO· resistance in *S. aureus*, a transposon-sequence study was conducted on the CA-MRSA strain, USA300 LAC [8]. This study was able to identify 168 genes that are thought to play a role in the resistance of NO·, some of which were selected for further study in the UNCA lab of Dr. Grosser. Two of particular interest are *ftsH*, a membrane bound ATP-dependent metalloprotease and *clpC*, an ATP-binding subunit of the ATP-dependent protease *Clp*. Both proteases have significant roles as it pertains to protein turnover, and both are known to affect several cellular mechanisms, such as stress response, metabolism, and virulence [9]. *FtsH* is an 'AAA'-type zinc metalloprotease that is attached to the bacterial cell membrane via two N-terminal transmembrane segments, functioning in the quality control and elimination of misfolded, misassembled, and unneeded proteins. *FtsH* occurs as a homohexameric protein, with its ATPase domain forming a hexameric ring with an axial pore [10]. Proteins destined for proteolysis are processed through this pore and into the proteolytic chamber, where they are degraded into smaller peptides. A previous study that examined the role of *ftsH* in *S. aureus* found that *ftsH* deletion mutants exhibited multiple defects including a significantly slower growth rate, sensitivity to multiple stressors, and a reduced ability to persist under starvation conditions [11]. Although there are multiple studies that corroborate the many defects present in *ftsH* mutants, information regarding its role in resistance to nitric oxide is lacking. *ClpC* is a member of the *Clp* proteolytic system and is thought to be the main degradation protease in *S. aureus* [9]. *ClpC* and other *Clp* proteases are critical for ensuring appropriate levels of misfolded and unfolded proteins within the cell when under stress conditions. The *clpC* subunit targets proteins for degradation through recognition of specific peptide sequences and additionally provides the energy needed for degradation [9]. A study on the role of *clpC* in biofilm-associated *S. aureus* infections determined that *clpC* mutants showed an increase in sensitivity towards oxidative and heat stress and a reduced expression of a gene (*citB*) involved in the tricarboxylic acid cycle, which resulted in both a loss of aconitase activity and catabolism of acetate throughout the stationary growth phase [12].

Due the fact that there has been little research done on how *ftsH* and *clpC* contribute to resistance of NO· in *S. aureus*, this research will focus on addressing those gaps of information through the creation of two separate deletion mutants using the CA-MRSA strain USA300 LAC. This research will provide a better understanding of how *S. aureus* is able to avoid the typical consequences that NO· causes during infection and will potentially provide new information that can be used in the future development of novel antibiotic therapies.

2. Materials and Methods

2.1. Mutagenesis Strategy

This research project utilized homologous recombination in the form of allelic exchange to generate chromosomal deletions of *FtsH* and *clpC* by replacing them with a kanamycin resistant marker, *kanR*. This was accomplished through PCR amplification of 1000 base pair flanking regions of both *ftsH* and *clpC*, which were further cloned into a plasmid on either side of the antibiotic resistance gene. The resulting plasmid was then transformed into a passage strain of *S. aureus*, RN4220, and was then subsequently transformed into the strain of study USA300 LAC. Mutants that were selected had successfully replaced *ftsH* and *clpC*, through homologous recombination, with the antibiotic resistant gene *kanR*. The methodology used throughout this process is described below (Figure 2).

2.2. Construction of Knockout Plasmid

Staphylococcus aureus MRSA strains USA300 and RN4420 were utilized as wild-type strains, and the *Escherichia coli* strain DH5α was used for shuttle plasmid creation. After the genes for testing had been identified, approximately 1000 base pair flanking regions both upstream and downstream of each gene were amplified with PCR using the primers found below in table one. This step ensures homology between each gene and its respective knockout plasmid. PCR was done using a T100 thermal cycler and NEB Q5 High-Fidelity DNA Polymerase. A touchdown protocol was performed (denaturation at 98ºC for 30 seconds, 25-35 cycles at 98ºC for 5-10 seconds, 50-72º for 20-30 seconds, 72ºC for 20-30 seconds per kilobase, final extension for 2 minutes at 72ºC, and hold at 4-10ºC) and the confirmation of PCR products were confirmed using gel electrophoresis. PCR products were purified using an IBI Gel/PCR DNA Fragments Extraction Kit. pBTK, a plasmid conferring kanamycin resistance, was isolated from cultured *E. coli* DH5α cells using the IBI High-Speed Plasmid Mini Kit. To confirm proper technique and DNA concentration, DNA concentration of the extracted pBTK plasmid was measured using the Gen 5 Microplate Reader.

The 5' flanking purified PCR fragment of each gene was digested with EcoRI and was then cloned into the EcoRI site of pBTK, creating the plasmids of interest pBTK_*ftsH*-5' and pBTK_*clpC*-5'. In order to prevent self-ligation of plasmid restriction sites, plasmids were treated with NEB Antarctic Phosphatase. A ligation protocol using the NEB T4 ligase protocol was performed, and the ligation product was transformed into the DH5α cells using the standard NEB heat shock transformation protocol. After the transformation was performed, cells were plated on LB agar +100µg/ml of ampicillin and incubated overnight at 37°C. To confirm the presence and directionality of the insert into the plasmid, multiple colonies were chosen from these plates and were subjected to a colony PCR protocol. The directionality of the insert was confirmed through the use of BT2.2a, a plasmid-specific forward primer purchased from Eurofin Genomics. Colonies were termed "successful" if the PCR product was measured at 1kb. Successful colonies were then grown in test tubes containing 5mL of LB (lysogeny broth) and 5µL of ampicillin overnight at 37°C. The following day, freezer stocks were prepared with 500µL of 50% glycerol and 500µL of each overnight culture and then stored in the -80°C freezer. The plasmids were extracted from these freezer stocks using the IBI High-Speed Mini Kit and DNA concentration was measured using the Gen 5 Microplate Reader.

Both 3' flanking regions of *ftsH* and *clpC* were then cloned into the BamH1 site of pBTK_*ftsH*-5' and pBTK_*clpC*-5' (Figure 1), using the same methodology described above. To confirm that the insert of interest was both present and directionally correct within the plasmid, a colony PCR was performed using a plasmid-specific reverse primer, BT2.2b, purchased from Eurofin Genomics. Colonies were then grown in lysogeny broth (LB) + Amp 100 overnight, and then were made into -80°C freezer stocks using 50% glycerol and 500µL of the liquid culture. The resulting plasmid was extracted from the above-described liquid culture using the IBI High-Speed Plasmid Mini Kit.

Table 1**.** List of all primers used throughout this study.

Primer Name	Sequence	Nucleotide Position
FtsH-5'-FOR	CACTAGAATTCGCTTACAGTGAGAACACGAC	+ / 2947
FtsH-5'-REV	CACTAGAATTCGTCATTTCCTCCTACTTCCC	- / 3932
FtsH-3'-FOR	CACTAGGATCCCGATCCAAATCACCCAGACA	+ / 6039
FtsH-5'-REV	CACTAGGATCCGAATCTCAGCCTCGCCCAAT	- / 7028
ClpC-5'.1a	CACTAGAATTCAGAAAATCATGTGCATCCAC	+ / 2866
ClpC-5'.1b	CACTAGAATTCGACCTCCTACTTTATATGTTC	- / 3742
ClpC-5'.3a	CACTAGGATCCACTAAAACACCATCGCAAGC	+ / 6222
ClpC-5'.3b	CACTAGGATCCCCACCAATAAGTAC	- / 6981

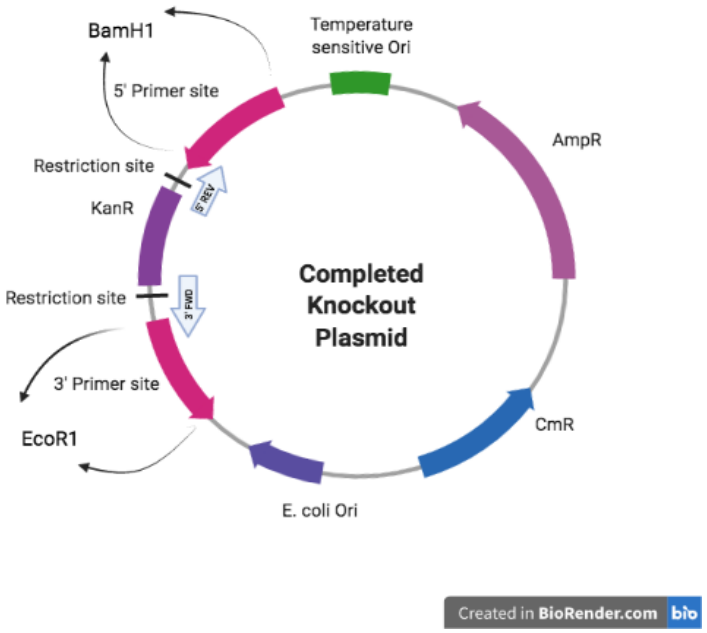

Figure 1. A diagram showing the complete knockout plasmid, now conferring kanamycin resistance.

2.3 *Deletion of ftsH and clpC from the S. aureus LAC genome*

In order to incorporate both previously generated knockout plasmids into the *S. aureus* strain of interest (*S. aureus LAC*), an intermediate strain known as *S. aureus* RN4220 was utilized. Most isolates of *S. aureus* prove very difficult when it comes to transforming exogenous DNA, especially from other species like *E. coli*, which is due to numerous restriction barriers within the genome of *S. aureus*. RN4220 is not able to properly restrict exogenous DNA, which allows an *E. coli*-based plasmid to be methylated properly and transformed into the target strain of *S. aureus* [13]. Using a technique described by Grosser et al., (2016), RN4220 electrocompetent cells and the miniprep product were combined and electroporated in order to prepare for transformation. The electroporated product was resuspended in B2 broth and placed into the incubator for approximately one hour. After incubation, the suspension was plated on TSB + Cm10 (10µM of chloramphenicol, or Cm10) plates and placed in the incubator at 30°C for 48 hours.

Colonies from the incubated plates were selected and grown in 5mL of TSB + Cm10 at 30°C. The product was then miniprepped using a modified version of the IBI miniprep protocol, which involved adding 12µL of lysostaphin to each miniprep after suspending with PD1 buffer. Now prepared appropriately to be taken up by the target strain, *S. aureus* LAC, the miniprepped product was transformed into LAC using the same electroporation procedure as described previously. The transformed cells were then plated on TSB + Cm10 and placed overnight in the incubator at 30°C. The next day colonies were selected and grown in TSB + Cm10 at 30°C overnight, and from this culture, a 1:1000 back-dilution was performed using 5mL of TSB, 5µL of overnight culture, and 5µL of chloramphenicol and placed in the incubator at 43°C. At the end of the day, each culture was subjected to a serial dilution and then plated on TSB + Cm10 at 43°C overnight. The next day colonies were selected and then grown in TSB + 100µM kanamycin (Kan100) at 30°C, no chloramphenicol was used in order to generate a second recombination event in the effort to remove the plasmid from the chromosome. The culture was passaged every day for three days by backdiluting each culture with TSB + Kan100.

After three consecutive days of passaging, a cycloserine enrichment was performed to select for kanamycin-resistant, chloramphenicol-sensitive clones that had successfully lost their plasmid. This process was done using a 1:1000 dilution of overnight culture with fresh TSB, which was then grown at 37°C for three hours. After the cultures had incubated the appropriate time, 5µL of Cm10 was added to stop cell growth in clones that were sensitive to Cm10. The cultures were left in the incubator at 37°C for thirty minutes, and then were inoculated with 5µL of cycloserine and left in the incubator for five hours at 37°C. Cycloserine only kills growing cells, so this step results in killing only cells that still retained the plasmid and continued to grow after the addition of Cm10. The resulting cultures containing the kanamycin-resistant, chloramphenicol-sensitive clones were then serially diluted (10^1 to 10^6) a second time and plated on TSB + Kan at 30°C overnight. All distinct colonies that had grown were then subsequently plated using a patching method onto TSB + Kan and TSB+ Cm10, which was done to select for Cm10-sensitive mutants. The process involved transferring a half or a whole distinct colony from the TSB + Kan plate with a pipette tip, transferring it to a plate containing TSB + Kan and then touching the same colony to a plate containing TSB + Cm10. To prevent mixing up colonies between the two plates, a grid was drawn on each plate and each square within the plate was numbered.

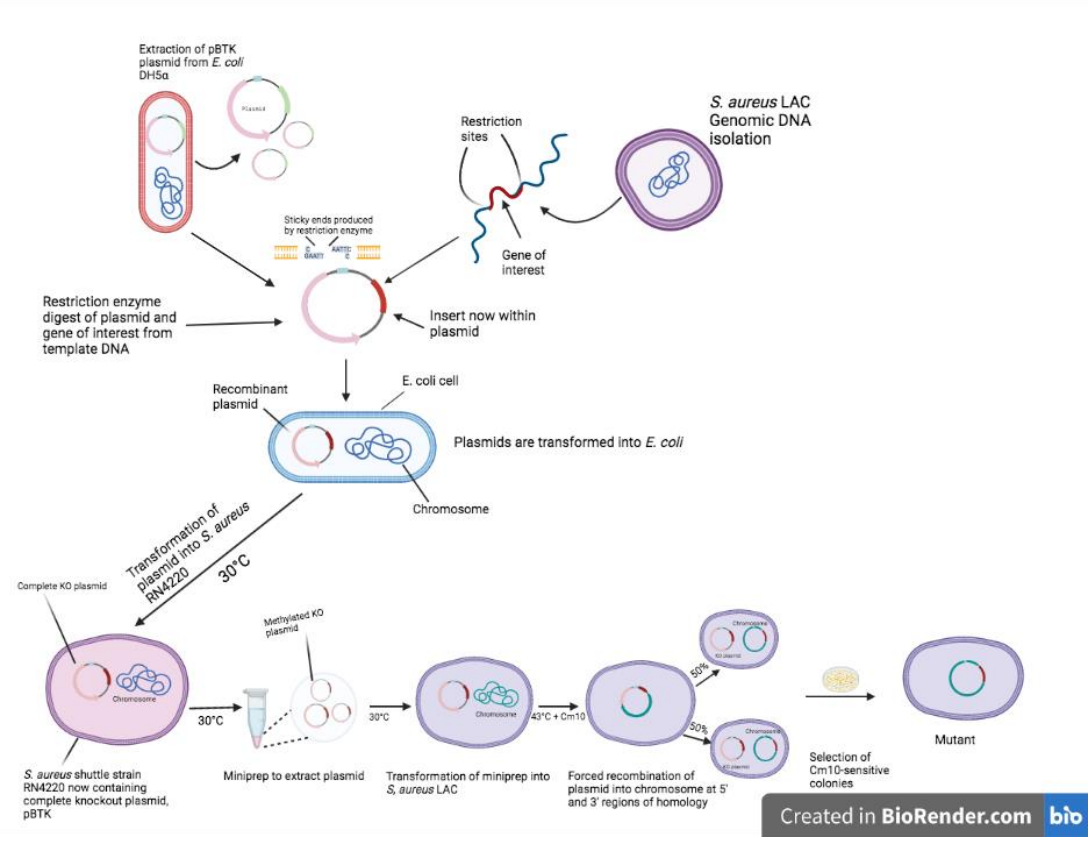

Figure 2. A schematic describing the methods utilized in this experiment to create deletion mutants. Flanking regions of *S. aureus* genes *ftsH* and *clpC* were amplified with PCR and then cloned into either side of a gene

conferring kanamycin resistance (*kanR*) found within the plasmid pBTK. After two rounds of restriction digestion and transformation into *E. coli*, the plasmid was transformed into the shuttle strain *S. aureus* RN4220. This strain is restriction deficient, which allows the strain to take up electroporated *E. coli* plasmids and methylate them for future uptake by *S. aureus* USA300 LAC. Methylated plasmids were then extracted from the resulting culture and transformed into the strain of study, LAC. Cells were subjected to incubation and back-dilution at both non-permissive and permissive temperatures and then plated to select for mutants that were kanamycin-resistant and. chloramphenicol-sensitive.

3. Results

In the effort to establish a better understanding of the genes that contribute to *S. aureus* resistance of exogenous NO·, a previous experiment using a genome-wide Tn-seq on the CA-MRSA strain USA300 was able to identify 168 genes of consequence [8]. Although these genes were identified as being required for fitness during exposure to NO· in a competition assay, their specific mechanisms are unknown and their requirements for fitness in pure culture was not yet established. To better understand this process, Δ*ftsH* and Δ*clpC* mutants were created via allelic exchange methodology. The generation of the two deletion mutants would further enable a comparison to the wild-type during nitric oxide stress.

3.1 Cloning in 3' and 5' inserts into *E. coli* DH5α

To characterize the mechanisms in which *S. aureus* resists host NO·, allelic exchange was performed to generate a knockout plasmid (Figure 1) by replacing the chromosomal copy of both genes of interest, *ftsH* and *clpC*, with a kanamycin resistance cassette (*kanR*). The plasmid, pBTK, was isolated from *E. coli* cells and the DNA concentration was measured at 44.0ng/µL. Approximately 1000 base-pair flanking regions both upstream and downstream of *ftsH* and *clpC* were amplified with PCR using isolated *S. aureus* LAC genomic DNA as a template and the primers listed in Table 1. This process was used to create regions of homology between the knockout plasmid and the chromosome, and to produce both 5' and 3' PCR products of each gene of interest. A first restriction digest of the 5' PCR products and the pBTK plasmid was performed using the restriction enzyme *EcoR1*. To prevent the plasmid from self-ligating, the 5' phosphate groups were removed using the enzyme phosphatase which reduces the occurrence of self-closure events during the future ligation process. The DNA concentration of both 5' products were measured and deemed successful prior to ligation, with *ftsH*-5' measuring at 39.1ng/µL and *clpC* measuring at 44.7ng/µL. A ligation and transformation of both 5' inserts into their respective plasmids was performed and colony PCR was utilized to confirm both the presence and the directionality of the insert. To verify the success of the colony PCR, a gel electrophoresis was performed on all resulting products. Colony PCR products were compared to a 10.0 kb ladder and were deemed successful if the product contained 1.0 kb (Figure 3). Among the 15 wells loaded, four were chosen (two from *ftsH* and two from *clpC*) to be used in the cloning of the second insert. A second restriction digest using the restriction enzyme *BamH1* was performed on the successful 3' PCR products described previously. The DNA concentration of both 3' products were deemed sufficient enough to proceed with digestion, with *ftsH*-3' measuring at 65.4ng/µL and *clpC*-3' measuring at 53.7ng/µL. After digestion, DNA concentrations were again measured to ensure success in the ligation and transformation process. The DNA concentration of *ftsH*-3' was measured at 19.0ng/µL and the concentration of *clpC*-3' was measured at 30.9ng/µL, both deemed appropriate levels to proceed. The ligation and transformation of both 3' products into *E. coli* DH5α was deemed successful through the use of colony PCR and gel electrophoresis (Figure 4).

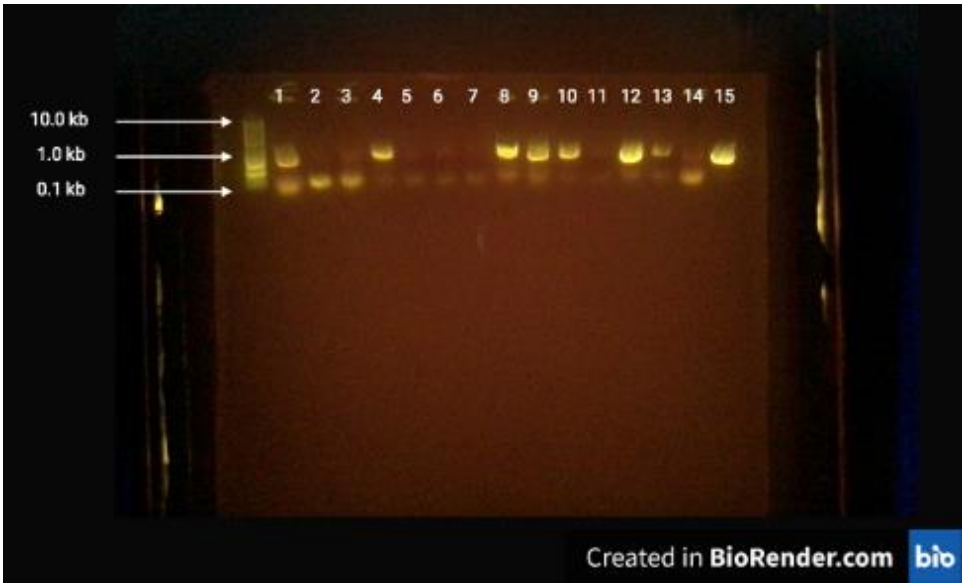

Figure 3. Gel electrophoresis performed showing successful transformation of 5' PCR products into *E. coli* DH5α electrocompetent cells. Lanes 1-10 correspond to pBTK_*ftsH*-5' products while lanes 11-15 correspond to pBTK_*clpC*-5'. A 1 kb DNA ladder purchased from NEB was utilized (far left lane) to confirm that products were the appropriate size to proceed, which in this case was 1.0 kb. *FtsH* lanes 4 and 8, and *clpC* lanes 12 and 15 were selected and corresponding colonies were grown overnight in a liquid culture of TSB + kan.

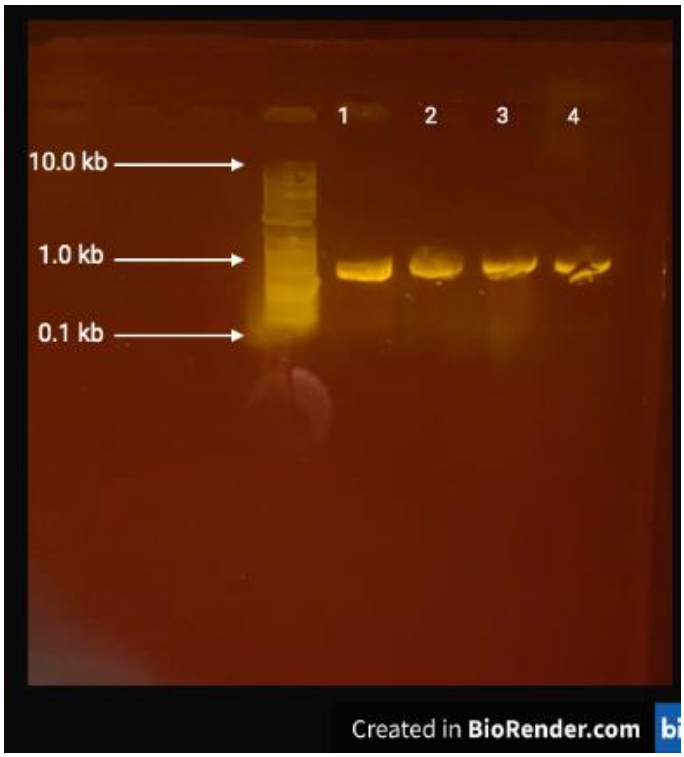

Figure 4. Gel electrophoresis performed following the second round of transformation into *E. coli* DH5α, which incorporated the *ftsH*-3' and *clpC*-3' inserts into the plasmid (pBTK + 5' insert) generated during the first round of cloning. The process was deemed successful due to the appearance of bands at the 1.0 kb mark on the DNA ladder (far left). Lanes 1 and 2 correspond to colonies containing the knockout plasmid pBTK_*ftsH*, while lanes 3 and 4

correspond to colonies containing the knockout plasmid pBTK_*clpC*. All four knockout plasmids were extracted from their corresponding colonies and transformed into the shuttle strain *S. aureus* RN4220.

3.2 Generation of Δ*ftsH* and Δ*clpC* mutants in USA-300 LAC

Plasmids were propagated via the *E. coli* shuttle strain, DH5α; plasmids now included the antibiotic resistant marker *kanR* and DNA sequences matching the flanking sequences of *ftsH* and *clpC*. The knockout plasmid now present within *E. coli* was miniprepped for extraction, then transformed into the restriction deficient strain *S. aureus* RN4220 through electroporation, miniprepped again, and the DNA concentrations were measured to ensure success. DNA concentration was measured at 38.8ng/μL for *clpC* and 53.2ng/μL for *ftsH*. Now successfully transformed into *S. aureus* RN4220, the resulting extracted plasmids were then subsequently transformed into the strain of interest *S. aureus* LAC. The non-permissive temperature utilized (43°C), and the regions of homology created between the flanking regions of both plasmids and the *S. aureus* chromosome allowed plasmid integration into the chromosome via a single recombination event. A second recombination event under permissive temperatures (30°C) allowed for the excising of each plasmid from their respective chromosome. This process facilitated the removal and replacement of both genes of interest, *ftsH* and *clpC*, with the kanamycin resistant cassette, *kanR*. To select for colonies that were sensitive to Cm10, cultures were treated with the bactericidal antibiotic cycloserine. After the cycloserine enrichment process, colonies were subject to patch-plating, which involved transferring individual colonies onto plates containing TSB + Kan and then quickly transferring them to a plate containing TSB + Cm10. Mutant colonies would have been able to grow on the plate containing kanamycin, but not chloramphenicol, which was unfortunately not observed in this case. Colonies grew successfully on both plates, which indicated that no resulting colonies were sensitive to kanamycin.

4. Discussion

NO· serves as a vital and effective component of host innate immunity during infection against a wide array of various pathogens. High levels of cytotoxic NO· produced by innate immune cells normally deliver a myriad of consequences towards invaders, including the alteration of metabolism and DNA replication, disruption of the electron transport chain, and targeting intracellular metal centers. While this hold true for the majority of invasive pathogens, *S. aureus* has evolved mechanisms to resist NO· and continue reproduction and growth within its host [7]. One of the main consequences of nitrosative stress, impaired respiration function, is avoided by *S. aureus* through the use of the two-component SrrAB system which serves to detect diminished respiration function and triggers the upregulation of various NO· resistance genes, and through an NO· inducible lactate dehydrogenase that enables the bacterial cells to metabolically adapt and replicate when respiration is impaired [14]. Although the resistance towards nitrosative stress that is seen in *S. aureus* is well documented, the mechanisms behind its resistance are poorly understood and studied. This study sought to better define NO· resistance mechanisms by selecting two genes of interest, *ftsH* and *clpC*, and to further determine how they assist in facilitating resistance.

While the knockout plasmid was successfully created and transformed into the final strain of interest, *S. aureus* USA300 LAC, there were issues surrounding the final step of cycloserine enrichment. At this point in the mutant making process, colonies needed to prove that they were sensitive to the antibiotic chloramphenicol in order to be considered a full mutant. Cycloserine enrichment serves to select for *S. aureus* colonies that are sensitive to the antibiotic chloramphenicol, while remaining resistant to kanamycin. To make the distinction between colonies that were sensitive to chloramphenicol and those that were not, colonies were patched onto a plate containing kanamycin and then subsequently patched onto a plate containing chloramphenicol. This first round of patch-plating proved unsuccessful, and a mutant was not observed. This result could be explained by a number of things. During the patch-plating process described above, there is a 50% chance that new colonies will be the wild-type strain and a 50% chance that colonies will be mutants, or less if the mutant contains a growth defect. In other words, generating a mutant strain is like playing a game of odds. In this case, increasing the number of colonies you plate during this process increases the odds of attaining a mutant. Another reason that could explain the difficulty in attaining a mutant during the first round of plating could hinge upon the two genes of interest in this study and their importance in cellular function. Functioning as ATP-dependent proteases, *ftsH* and *clpC* play important cellular roles in the quality control of proteins. A recent study that sought to define the mechanisms of *S. aureus* cell aggregation that is observed in Δ*ftsH* mutants found that a total of 277 genes were transcriptionally affected when the gene was no longer within the genome [10]. This

implies that *ftsH* might have an extensive impact on cellular function, especially as it pertains to post-transcriptional regulation. While this evidence lends itself to the idea that *ftsH* is important for normal cellular function and therefore generating a Δ*ftsH* mutant could prove difficult, it is still very possible according to the study mentioned above.

ClpC also plays an important role in cellular function through metabolism regulation, oxidative stress response, and cell survival; but has also been successfully deleted from the *S. aureus* genome in many previous studies [15].
Previous research shows that generating Δ*ftsH* and Δ*clpC* mutants is certainly possible, therefore the patch-plating procedure is currently being repeated. After a mutant is successfully generated and verified using Q5 PCR and gel electrophoresis, 24-hour growth curves will be utilized in order to compare growth phenotypes in the deletion mutants. The growth curves will be performed using a 96-well plate and select wells will be treated with DETA/NO, slow-release NO· donor. This procedure serves to compare growth between the mutant LAC and the wild-type LAC under various concentrations of NO·, and also allows a comparison to be made on the recovery times (time to reach exponential growth) between each phenotype. Nitrosative stress has been shown in previous studies to both induce and potentiate oxidative stress, so further testing will employ the use of hydrogen peroxide, as it is known to cause oxidative stress to bacterial cells[16].

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6. References

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