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Two-Pronged Approach to Investigating Regulation of the NO· Stress Response in *S. aureus*

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Abstract

Methicillin-resistant *Staphylococcus aureus* is a Gram-positive bacterium and an opportunistic pathogen. It causes infections such as skin abscesses, sepsis, urinary tract infections, and meningitis. Due to antibiotic resistance*, S. aureus* is a major public health concern. One of the contributing factors to its survival in a host is cyclic di-adenosine monophosphate (c-di-AMP). This second messenger regulates many aspects of cellular function in Gram-positive bacteria, including maintaining osmotic pressure and cell wall homeostasis. In *S. aureus*, an enzyme called DacA generates c-di-AMP from two ATP molecules. C-di-AMP is degraded into 5'-pApA by the phosphodiesterase enzyme GdpP. The deletion of DacA causes growth arrest and sensitization to antibiotics, which indicates the importance of c-di-AMP for bacterial survival. Most c-di-AMP quantification methods require purification of cell lysates; however, to understand how dynamic changes in c-di-AMP are related to antimicrobial resistance and stress responses, it will be useful to have a fluorescence-based biosensor that can be monitored in real-time in live cells. In this project, we are adapting and testing a fluorescence resonance energy transfer (FRET)-based c-di-AMP biosensor created for *Listeria monocytogenes* for use in *S. aureus*. The biosensor includes cyan fluorescent protein (CFP) and yellow fluorescent protein (YFP) linked by a truncated c-di-AMP binding protein (lmo0553) from *L. monocytogenes*. When c-di-AMP is present at high levels, lmo0553 changes conformation to bring CFP and YFP into close proximity, allowing FRET and a change in emission spectra to occur. We are testing the expression of the biosensor from the *S. aureus* plasmid pCN52, with expression driven by either the constitutive *rpsJ* or *rpoD* promoters. The resulting vector will be tested in *S. aureus* strains overexpressing DacA or GdpP and in conjunction with an ELISA to calibrate a standard curve relating FRET results to c-di-AMP concentration.

1. Introduction

Each year *Staphylococcus aureus*, a highly invasive, Gram-positive bacterium, is the cause of a significant rate of global morbidity (Suaya et al. 2014). The tendency of S. *aureus* to swiftly develop antibiotic resistance has made treating S. *aureus* infections more challenging. Methicillin-resistant S. *aureus* (MRSA) and multidrug-resistant MRSA clones are widespread. Although historically a nosocomial disease, MRSA has spread outside hospitals in recent years, even in otherwise healthy people. These community-acquired MRSA strains are commonly responsible for skin and soft tissue infections (SSTIs) and are typically described as hypervirulent. These infections, however, frequently develop into more serious, systemic disorders like sepsis and meningitis (Thurlow et al. 2012).

Nitric oxide ($NO\cdot$) is a mammalian innate immune effector and is produced from L-arginine by nitric oxide synthase (NOS) enzymes, making a free radical and citrulline as a byproduct (Geller & Billiar 1998). $NO\cdot$ is a membrane-permeable, antimicrobial gas and a respiratory inhibitor that is generated by the host's stimulated phagocytes during bacterial infection (Ribeiro et al. 2021). It increases bacterial susceptibility to antibiotics and slows antibiotic resistance when used in conjunction with traditional antibiotics. Antibiotic resistance has become more common in recent years, presenting a public health concern (Carlet 2014). Antibiotics have become less effective against their target due to overuse and the bacteria's ability to develop tolerance.

S. aureus exhibits remarkable resistance to numerous components of the host immune response, including $NO\cdot$ (Grosser et al. 2018). A previous Tn-Seq study in a community acquired-MRSA strain (USA300 LAC), identified 168 genes that were likely to play a role in $NO\cdot$ resistance in S. aureus (Grosser et al. 2018). One of these genes, likely to influence NO resistance, is mgrA, a multiple antibiotic-resistant regulator family (MarR). The transcriptional members of the MarR family that code for DNA-binding proteins are dimers that bind palindromic sequences and a variety of ligands. MarR family proteins with winged helix motifs linked to DNA binding are frequently found in prokaryotes with large genome sizes, including bacteria and archaea. The various MarR regulators control a wide variety of genes, including those involved in responding to antimicrobial stress. The cellular processes oxidative stress, antibiotic resistance, resistance to organic solvents, resistance to household cleaners, and resistance to organic solvents are all regulated by additional MarR family members (Grove 2017; Haque et al. 2009). They also control how virulence components are made by microorganisms infecting people and plants. Because MarR family regulators typically participate in responses to environmental conditions, they are remarkably effective at controlling the virulence genes of pathogenic bacteria like S. aureus. For instance, the transcriptional regulators known as MarR are known to control an operon that encodes a drug efflux pump in *E. coli*. This holds true in *S. aureus* as well because *mgrA* has the ability to control efflux pumps. An earlier investigation revealed that the 147-amino-acid protein MgrA controls the expression of the efflux pump-encoding genes NorA, NorB, and tet38, all of which are known to increase resistance to a number of antibiotics (Chen et al. 2006; Truong-Bolduc et al. 2005).

Our lab previous found that a Δ*mgrA* deletion mutant exhibited a faster recovery time in the presence of $NO\cdot$. MgrA is a repressor of many genes and likely overexpresses specific genes in the Δ*mgrA* mutant, some of which may be beneficial, potentially explaining its faster recovery

time. The first part of my project further investigated different *mgrA* mutants (Table 1.) made by former lab member David Olawuni, including new strains in which *mgrA* expression is added back on an inducible plasmid. We explored MgrA's role in NO· resistance by running various growth curves. We predicted that the overexpression strain LAC pRMC2 – *mgrA* will behave similarly as ΔMgra + PRMC2 – *mgrA* (the complemented deletion mutant).

The previously conducted transposon screen (Grosser et al., 2018) also identified three genes associated with cyclic di-adenosine monophosphate (c-di-AMP) signaling as important for NO· resistance. This second messenger regulates many aspects of cellular function in Gram-positive bacteria, including maintaining osmotic pressure and cell wall homeostasis (Figure 1). In *S. aureus*, an enzyme called DacA generates c-di-AMP from two ATP molecules (include citations for this and the next few sentences). C-di-AMP is degraded into 5'-pApA by the phosphodiesterase enzyme GdpP. The deletion of DacA causes growth arrest and sensitization to antibiotics, which indicates the importance of c-di-AMP for bacterial survival. Preliminary data from our lab suggests that the c-di-AMP signaling network may also represent a target for disrupting NO· resistance. For example, overexpression of DacA results in inhibited growth during NO· stress. Therefore, we are particularly interested in characterizing how *S. aureus* normally maintains its c-di-AMP levels during NO· stress.

Most c-di-AMP quantification methods such as liquid chromatography or liquid chromatography-mass spectroscopy require the purification of cell lysates; however, to understand how dynamic changes in c-di-AMP are related to antimicrobial resistance and stress responses, a fluorescence-based biosensor would be beneficial because it can be monitored in real-time in live cells. By adapting a fluorescence resonance energy transfer (FRET)-based c-di-AMP biosensor created for *Listeria monocytogenes* for use in *S. aureus*, we aim to quantify c-di-AMP (Figure 2). Thus, the second part of my project involved constructing a potential biosensor that includes cyan fluorescent protein (CFP) and yellow fluorescent protein (YFP) linked by a truncated c-di-AMP binding protein (lmo0553) from *L. monocytogenes*. When c-di-AMP is present at high levels, lmo0553 will change conformation to bring CFP and YFP into close proximity, allowing FRET and a change in emission spectra to occur. Our research will test the expression of the biosensor from the *S. aureus* plasmid from pCN52, with expression driven from either the constitutive *rpsJ* or *rpoD* promoters. In the future, the resulting vectors from my work will be tested in *S. aureus* strains overexpressing DacA or GdpP, and in conjunction with an ELISA to calibrate a standard curve relating FRET results to c-di-AMP concentration.

Table 1. List of mutant strains used in this study.

LAC pRMC2
LAC pRMC2 – *mgrA*
ΔmgrA + PRMC2
ΔmgrA + PRMC2 - *mgrA*

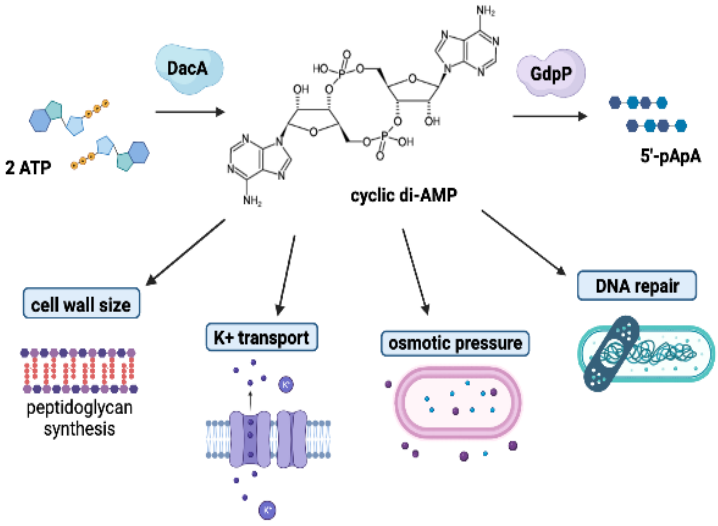

Figure 1. Model portraying some cellular processes regulated by c-di-AMP as well as synthesis and degradation.

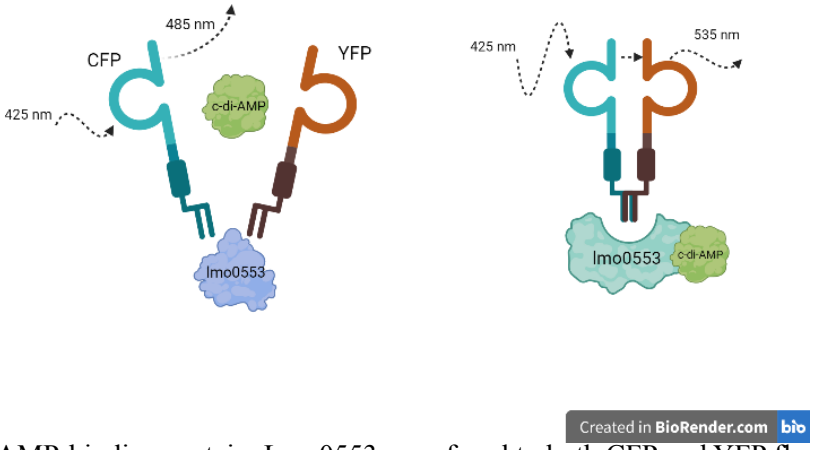

Figure 2. The c-di-AMP-binding protein, Lmo0553, was fused to both CFP and YFP fluorescent proteins. When Lmo0553 binds to c-di-AMP, it alters its shape so CFP and YFP are brought close together. The CFP excitation (425nm) and emission (485nm) of energy will transfer to YFP when in close proximity to one another resulting in an overall emission (535).

2. Materials and Methods

2.1 Growth curves with NO·

To compare growth phenotypes of the Δ*mgrA* mutant relative to wild-type, 24-hour growth curves were generated under a variety of conditions using 96-well plates, where each well serves as an independent

culture. To prepare bacteria for growth curves, the four knockout or overexpression strains (Table. 1) were grown in 5ml TSB overnight (O/N) and incubated at 37°C and 250rpm of shaking for 24hrs. The culture optical density (OD) was measured at 650nm and was diluted to a final OD650 of 0.01 for each strain in fresh TSB. We transferred 200ul of each culture into wells of a 96-well plate. Select wells were inoculated with 5mM or 10mM DETA/NO (a slow-release NO· donor), and growth curves were generated using the BioTek biology plate reader to track absorbance (a proxy for growth) at 15-minute intervals (Growth OD_{650}: 37°C, 97 kinetic cycles; First shaking: 830 seconds shaking, orbital mode, 1mm amplitude; 2nd shaking: 30 sec duration, linear mode, 1mm amplitude, 10 sec settle time, 5 flashes).

2.2 Growth curves in PN media

To further analyze the mutant plasmids NO· resistance, a defined minimal media (PN media) was made, to replace TSB (Table 2.). TSB contains heme, which can scavenge NO· and potentially alter bacterial exposure during the growth curve. PN media does not contain heme or other NO· scavengers and circumvents this potential factor. A 50ml stock was prepared prior to each growth curve. The media was then pH balanced to 7.4 and supplemented with glucose at 5mg/ml. PN media is usable for 3 days at room temperature. Media will support growth for longer than 3 days, but growth rates become unreliable and often decline after this time period.

Table 2. PN media Ingredients.

Ingredients	X-Stock	Stock (mg/mL)
Salts	**100**	
K2HPO4		70
KH2PO4		20
(NH4)2SO4		10
Amino Acids	**100**	
Phe		4
Iso		3
Tyr		5
Cys		2
Glu		10
Lys		1
Met		7
His		3
Trp		1

Leu		9
Asp		9
Arg		7
Ser		3
Ala		6
Thr		3
Gly		5
Val		8
Pro		1
Bases	**100**	
Adenine		.5
Cytosine		.5
Guanine		.5
Thymine		2
Uralcil		.5
Individual Solutions		
MgSO4 7H2O	100	5
FeCl3	1000	8
Vitamin Solution	**1000**	
Thiamine		1
Niacin		1.2
Biotin		.005
Ca Pantothenate		.25

2.3 Construction of biosensor

To construct a biosensor that would be highly expressed in *S. aureus*, two constitutive *S. aureus* promoters (*rpoD* and *rpsJ*) were cloned into the *S. aureus* vectors pRMC2 and pCN52. These plasmids were then extracted from *E. coli* by mini-prepping using IBI scientific (Iowa) protocol on the *E. coli* PCN52-*rpsJ* and PCN52-*rpoD* overnight cultures. Next, an enzyme digest was performed on the miniprepped plasmids using Kpn1 and Pst1 enzymes. A PCR cleanup was then performed on the resulting digest using IBI scientific protocol (Iowa). The CDA5 (FRET) biosensor sequence was modified from the *Listeria monocytogenes* version (Pollock et. al 2021). The FRET reporter was synthesized via TWIST Biosciences in the pTwist vector, then restriction digest and gel extraction were used to purify the reporter DNA fragment (cut from the vector using Kpn1 and Pst1). The purified fragment was cloned behind either the constitutive *S. aureus rpsJ* or *rpoD* promoter into the KpnI/PstI cut sites of the plasmid pCN52. A confirmation gel was performed to ensure we had the correctly-sized insert as well as Sanger Sequencing (Genewiz) to confirm the sequence integrity of the insert. P066 and P067 primers were used in PCR to amplify around 900kb (pRMC2 plasmids). P068 and P069 were used for the 2kb plasmid (pCN52). The sequence we received from Sanger Sequencing was analyzed in Benchling and aligned with our expected plasmid sequence.

Table 2. List of primers used.

Name	Sequence (5'-3')
P011	AAACTATGTAGCATCACCTTCACC
P054	CACTAGAATTCGAAAATAGTTGAACTGACTAAG
P056	CACTAGAATTCTATGTCAAAATGTATAAGGG
PO66	GGCGCCGGGCCCCCTTTGCGGAAAGAGTTAATAAG
P067	CGAGTTCATGAACCAATAAAAGCAATCAATGAAC
P068	TTGCTTTTATTGGTTCATGAACTCGAGGGGATC
P069	AATTATAGCACGAATCGACACTGAATTTGCTC

2.2 Transformation into *S. aureus*

Electroporation was performed on the resulting biosensor plasmid into *S. aureus* 'RN4220', a strain which has a mutation in the sau1 hsdR gene, resulting in a restriction deficiency, which facilitates the uptake of *E. coli* DNA. This strain methylates incoming *E. coli* DNA appropriately for *S. aureus*, which then allows for uptake by other *S. aureus* strains (Nair et al, 2011). RN4220 was made electrocompetent using a well-established procedure and electroporated according to the procedures outlined by Grosser et al., 2016. Briefly, O/N cultures of *S. aureus* in B2 Broth were diluted 1:100 in fresh B2 broth and grown to mid-log (OD_{660} = ~0.8). The cells were then washed with room temperature (RT) ddH_2O, RT 10% glycerol and later resuspended in RT 10%

glycerol. Once cells were at this stage, DNA was mixed in and samples were transferred to pulse cuvette (BioRad 165-2086) and pulsed at 1.8 kV, 600 Ω, 10 µF. Following electroporation, cultures were plated on Tryptic Soy Agar (TSA) plates with 5µg/ml of the antibiotic chloramphenicol (Cm5) and then placed in an incubator overnight at 37°C. Successful colonies were chosen and grown in a test tube containing 5.0 mL of liquid media Tryptic Soy Broth (TSB) and 5.0µL of Cm 5, and placed in an incubator overnight at 37°C.

2. Results

3.1 MgrA growth curve results

Our goal was to test four *mgrA* mutant and overexpression strains under NO· stress. We performed various growth curves in 96-well plates using the NO· donor DETA/NO. As an approximation of lag time, we calculated the time for each strain to reach the exponential phase (OD650 0.5) during aerobic growth and NO· stress. Despite observed differences in strain phenotypes within individual replicates, our overall data was highly variable (Figure 5). We expected the knockout plasmid to increase lag time compared to the wild type (WT), however, this was not always the case, and in some replicates, we found that overexpression of *mgrA* also increased the lag time. We also did not expect such significant variability between replicates. Some replicates we observed suggested that the presence of too much MgrA is detrimental to *S.aureus* survival (as well as its absence) but the results varied and were inconsistent (see examples of two separate biological replicates in Figure 3,4). We did not observe substantial differences in results between experiments conducted in TSB versus PN media.

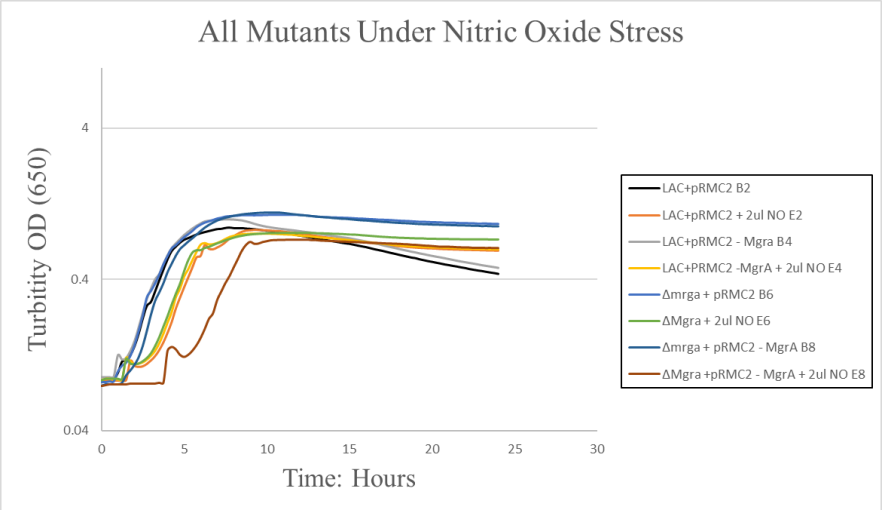

Figure 3. 24-hour growth curve representation (from a single biological replicate) of four mutant strains under normal and stress conditions in TSB. ΔmgrA + PRMC2 – *mgrA* has a significantly increased lag time compared to other mutant strains.

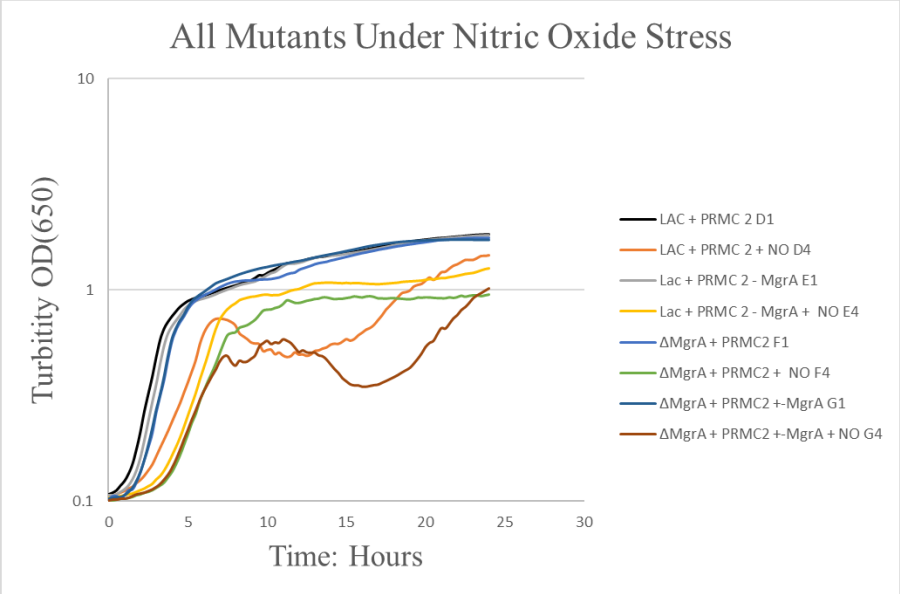

Figure 4. Another 24-hour growth curve (from a different biological replicate) represents four mutant strains under normal and stress conditions in TSB. The overexpression of *mgrA* as well as the deletion of *mgrA* increases the lag time compared to WT.

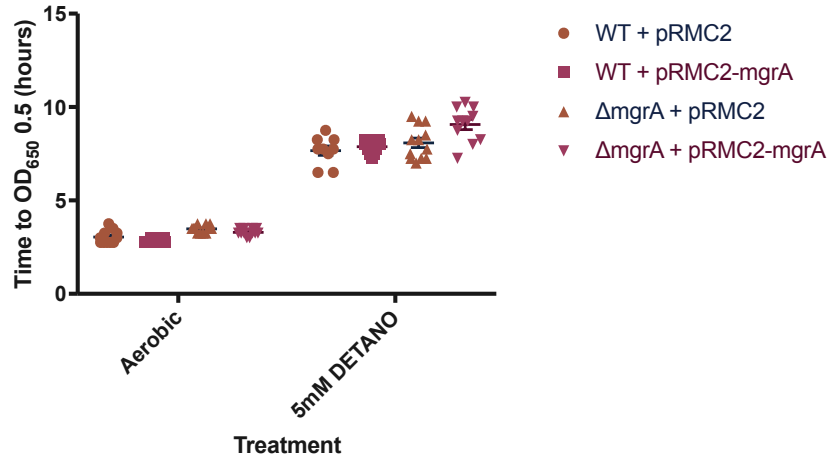

Figure 5. Analysis of lag time between mutant strains under normal and stress conditions. There is no significant difference in lag time between the mutant strains. Variability is seen across replicates.

3.2 Biosensor construction results

Our next goal was to construct a biosensor in *S. aureus* to quantify cyclic-di-AMP. To construct a FRET reporter plasmid for *S. aureus*, we first had the reporter gene fragment synthesized in a stock plasmid by TWIST Biosciences. Several attempts to PCR amplify the reporter fragment from the plasmid failed. This may have been from YFP and CFP being too close in sequence to successfully design flanking primers that annealed only once to the biosensor fragment. Instead, the fragment was cut out of the plasmid with Kpn1 and Pst1, (plus the addition of Fsp1to cut the remaining plasmid backbone in half so it would not be the same size as the desired insert). The

biosensor fragment was gel extracted (Fig. 6A), and then ligated into our PCN52 plasmid downstream of either *rpsJ* and *rpoD* constitutive promoters (Fig. 6C), and transformed via heat shock into *E. coli* Dh5a chemically competent cells. To determine if we had cloned the right part of lmo0553 and no other areas of the CDA5 plasmid into the pCN52 vector, we digested colony mini preps with KpnI/PstI and ran a confirmation gel, which showed validation of an insert with size matching the FRET biosensor (Figure 6D). We determined this because the bands were around the 2kb marker, which was the expected size of the cut plasmid's insert. The plasmid was also sent off for Sanger Sequencing at Genewiz for sequence confirmation. Out of the ten plasmids sent to sequencing, only two resulted in perfect alignment with our expected sequencing from Benchling.

After obtaining the final biosensor plasmid, we next tried to transform it into *S. aureus*. After many attempts, the transformation of the plasmid into *S. aureus* strain RN4220 was ultimately unsuccessful. After plating transformed RN4220 on TSA + chloramphenicol plates, colonies grew on the plate, however, no growth was found in the overnight cultures attempted from these colonies. Multiple methods were used to try to correct this error including making new plates and re-electroporation, but we were unsuccessful.

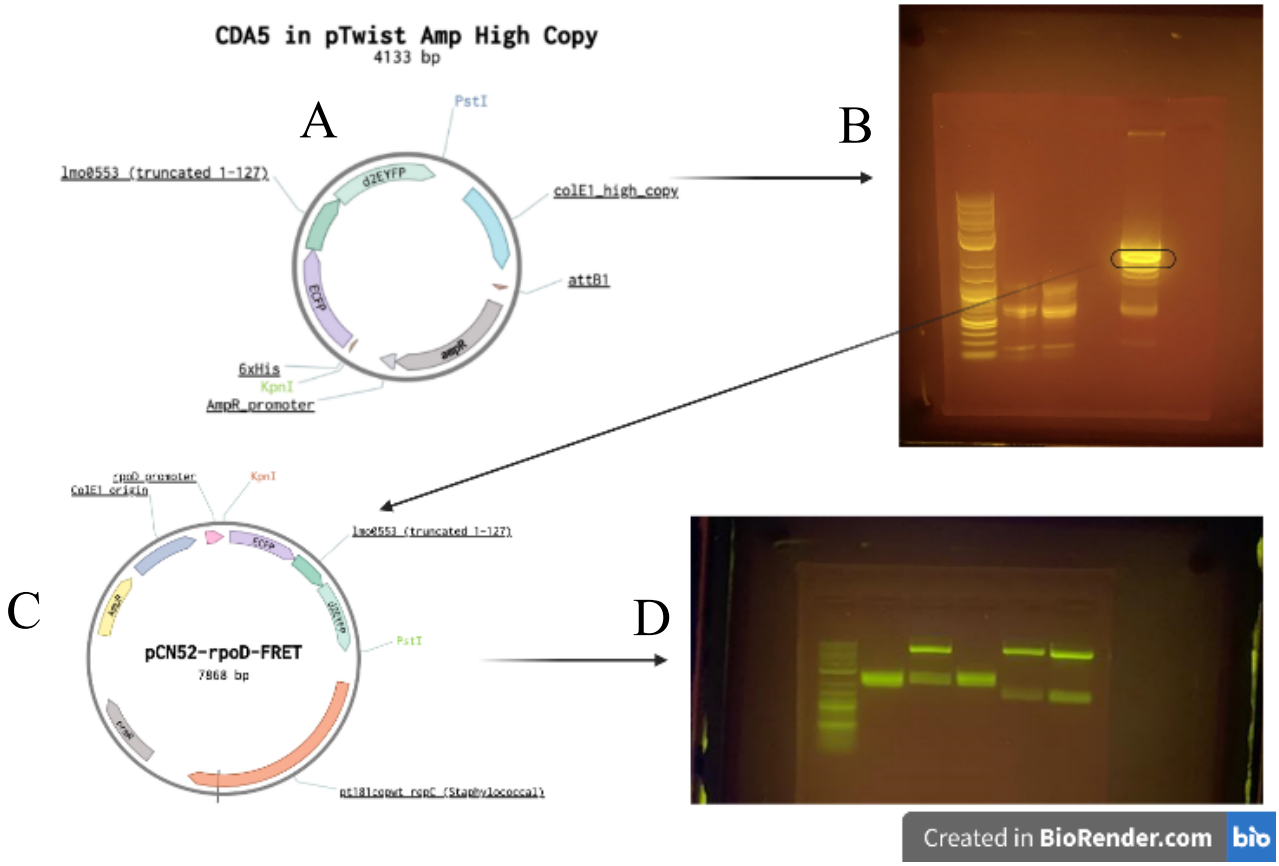

Figure 6. An adapted FRET plasmid was created for *S. aureus* (C). The FRET reporter was synthesized via TWIST Biosciences in the pTwist vector (A), then restriction digest and gel extraction (B) were used

to purify the reporter DNA fragment (cut from the vector using Kpn1 and Pst1). The purified fragment was cloned behind either the constitutive *S. aureus rpsJ* or *rpoD* promoter in the plasmid pCN52. A confirmation gel depicting two plasmids *(rpoD*2-1 and *rpoD*2-2) with PstI/KpnI digest bands, representing the FRET insert, around the 2kb mark (D).

4. Discussion

Due to its antibiotic resistance and virulence, *S. aureus* can combat many antimicrobial attacks from the human immune system (Grosser et al. 2016; Cheung et al. 2021). We performed growth curves to analyze four MgrA mutant and overexpression strains under nitric oxide stress. We expected the MgrA overexpression would cause *S. aureus to* perform worse under stress because the ΔmgrA deletion mutant performs better than WT under stress. The results were unexpected since the more growth curves we ran, the more inconclusive and varied the results were. We used a minimal PN media as well to see if controlling the amount of nutrients *S. aureus* can use would change the results. Variability could be inherent to the activity of MgrA, which is a transcriptional regulator and may not act uniformly across populations due to biostability in regulation. There might also be a stress threshold necessary for MgrA activation that is not always reached during our assays. To combat this, we may need to change NO· concentrations. The presence of the pRMC2 plasmid (even the empty vector) plus the addition of inducer (aTc) may change growth dynamics compared to growth curves performed by previous students as well. For future work, we will perform RNA-Seq to investigate gene expression changes when MgrA is absent or over-expressed during NO· stress.

C-di-AMP, a second messenger, plays a significant role in *S. aureus* pathogenesis. In the second part of this project, we created a biosensor that we expect will detect levels of cyclic-di-AMP in *S. aureus.* Although the transformation of the final plasmid into *S. aureus* was unsuccessful, we will be trying a different approach. The issue could be either molecular (an error occurring in the digest or ligation) or microbiological (competent cell preparation). We have successfully prepared new plasmid preps from *E. coli* and a new ligation of the FRET biosensor plasmid; however, we still need to perform a confirmation gel for the colonies and sequence before transforming into *S. aureus*. One possibility is that the overexpression of the FRET biosensor from the pCN plasmid and constitutive *rpoD/rpsJ* promoters is toxic to *S. aureus* or causes an excessively large metabolic burden. A lower copy vector or less highly expressed promoter may help with this issue for biosensor expression. For future work, once we successfully express the biosensor in *S. aureus*, we will use an ELISA-based assay to calibrate the FRET-based biosensor for c-di-AMP, another means of measuring c-di-AMP in *S. aureus* in real-time. Long-term, these findings may contribute to the development of drugs that alter the signaling pathways of antibiotic-resistant bacteria. Overall, our findings show that creating a Cyclic-di-AMP biosensor is possible, but we must optimize our approach to successfully transfer the plasmid into *S. aureus*.

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BioRender.com was used for the creation of schematics.

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