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Quantifying cyclic di-AMP in *Staphylococcus aureus* during stress using a competitive ELISA

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Abstract

Staphylococcus aureus, a Gram-positive bacterium*,* is one of the leading causes of life-threatening bacterial infection in the US, partly due to its antimicrobial resistance. Diseases like pneumonia, skin and soft tissue infections, sepsis, and endocarditis may arise from a serious staph infection. *S. aureus* uses a second messenger called cyclic di-adenosine monophosphate (c-di-AMP) to regulate many cellular functions such as peptidoglycan synthesis, stress response, and more. Our data show that high c-di-AMP levels can be toxic during nitric oxide (NO·) stress. Others have shown that low c-di-AMP levels result in reactive oxygen species (ROS) toxicity in *S. aureus*. Therefore, maintaining normal c-di-AMP is essential for proper cell functions, especially during stress. In this research, we aim to optimize a competitive enzyme-linked immunosorbent assay (ELISA) to quantify specific concentrations of c-di-AMP in *S. aureus*, with the ultimate goal of understanding how c-di-AMP levels change during stress and what levels are optimal. For assay development, we used three strains: *S. aureus* containing a vector control of an anhydrotetracycline (aTc)-inducible plasmid (pRMC2), and *S. aureus* overexpressing the genes encoding either DacA (produces c-di-AMP) or GdpP (hydrolyzes c-di-AMP) from pRMC2. Growth conditions and lysate purification methods were tested with these strains in conjunction with a commercial c-di-AMP ELISA kit, and a protocol was developed to successfully detect different concentrations of c-di-AMP in *S. aureus*. Currently, we are using this new assay combined with stepwise induction of DacA or GdpP via aTc (in the above strains) to determine the precise concentration range of c-di-AMP that can be tolerated in the presence of stressors. We are also using ELISA to calibrate a fluorescence resonance energy transfer (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 signaling pathways of antibiotic-resistant bacteria.

1. Introduction

Staphylococcus aureus is a pathogenic, Gram-positive bacterium responsible for moderate skin infections to life-threatening diseases such as sepsis, endocarditis, and osteomyelitis (Tong et al. 2015; Guo et al. 2020). The virulence factors of *S. aureus* enable the bacteria to survive host immune responses and other extreme conditions (Grosser et al. 2016; Kong et al. 2016). For example, when subjected to the radical nitric oxide (NO·), an antimicrobial gas produced by the innate immune response upon infection, *S. aureus* can continue to grow whereas other bacterial species are inhibited (Grosser et al. 2018). NO· is a product of stimulated phagocytes that inhibits bacterial growth and replication by disrupting bacterial respiratory processes (Grosser et al. 2018). NO· as a radical allows it to interact with oxygen to produce toxic reactive nitrogen and oxygen species (RNS/ROS) that destroy bacterial DNA and lipids and target metal centers and cofactors of bacterial enzymes to promote cell death (Grosser et al. 2016). With its ability to adapt and resist NO·, Methicillin-resistant *Staphylococcus aureus* (MRSA), is one of the world's leading infectious agents, causing more morbidity and mortality in 2018 than any other antibiotic-resistant bacteria (Grosser et al. 2018; Guo et al. 2020; Cheung et al. 2021). The prevalence of antibiotic-resistant strains has made the treatment of *S. aureus* infections increasingly challenging, highlighting the need for novel therapeutic approaches (Guo et al., 2020; Cheung et al., 2021).

One potential target for such approaches is the signaling network controlled by c-di-AMP, a second messenger molecule that plays a crucial role in bacterial growth, physiology, and virulence (Corrigan et al., 2011; Fahmi et al., 2017; Zeden et al., 2018). This second messenger can regulate cell wall maintenance, DNA damage repair, transport of essential ions like potassium, and other intracellular processes (Corrigan et al. 2013; Bowman et al. 2016; Fahmiet et al. 2017). In *S. aureus* and other Gram-positive bacteria, c-di-AMP allows cells to adapt under stressful conditions (Corrigan et al., 2011; Fahmi et al. 2017; Zeden et al., 2018). This second messenger is synthesized by a protein called DacA which links two ATP molecules . In addition, it can be hydrolyzed into 5'-pApA by GdpP, a phosphodiesterase enzyme (Corrigan et al., 2011; Fahmi et al. 2017; Bowman et al., 2016). A knock-out of the protein DacA can cause growth arrest and bacterial sensitization to antibiotics (Zeden et al. 2018). Therefore, the importance of DacA to promote an optimal level of c-di-AMP is required for survival (Zeden et al. 2018). Interestingly, other studies have discovered that a drop in c-di-AMP can trigger biofilm formation (Fahmi et al. 2017; Syed et al. 2020). However, if c-di-AMP levels are too low in *S. aureus*, reactive oxygen species (ROS) toxicity rises in cells. A transposon-sequencing screen (Tn-Seq) found that three genes associated with c-di-AMP signaling are critical during NO· stress, suggesting that maintaining normal c-di-AMP levels is especially important during NO· stress (Corrigan et al., 2011; Grosser et al., 2018; Zeden et al., 2018).

There remains a need to understand the optimal level of c-di-AMP for *S. aureus* in the presence of stressors. Here, we performed growth curves with stepwise increases in DacA during NO· stress to determine the level of overexpression where we first see toxicity due to elevated c-di-AMP. Next, we aimed to determine the precise concentrations of c-di-AMP corresponding to each level of DacA induction. Various methods to study and quantify c-di-AMP have been developed, including a competitive enzyme-linked immunosorbent assay (ELISA) that can detect c-di-AMP in bacterial lysates and culture supernatants. ELISA uses antibodies that specifically recognize c-di-AMP, providing a valuable tool for quantifying levels of this molecule and studying its regulation and function (Underwood et al. 2014). To determine intracellular concentrations of c-di-AMP, we optimized a commercial ELISA

kit with different controls of stepwise induction via anhydrotetracycline (aTc, an inducible promoter) on three plasmid strains of *S. aureus* overexpressing DacA and GdpP under stressful conditions.

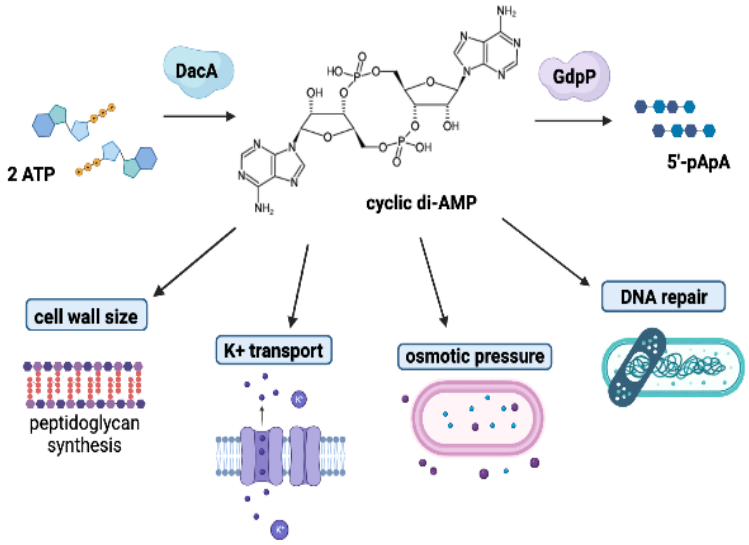

Figure 1. Model portraying some cellular processes regulated by c-di-AMP such as cell wall size, potassium (K+) transport, osmotic pressure, and DNA repair.

2. Materials and Methods

2.1 Growth Curve Assays

Phenotypic growth comparisons were performed on the WT-LAC pRMC2 vector control and WT-LAC pRMC2-dacA strains. Growth curves were performed over a 24-hour interval under a variety of induction conditions using 96-well plates and a BioTek Synergy H1 microplate reader to analyze the individual wells. For the growth curve, cultures of bacteria were prepared by inoculating and growing each strain individually overnight with 5.0 mL of TSB and 5µL of 10 mg/L chloramphenicol antibiotics (Cm10). Then, the cultures were shaken and incubated at 37°C for 24 hrs. The following day, the OD of each culture sample was measured at a wavelength of 650 nm before being diluted to a final OD_{650} of 0.01. Next, 200µl of each sample was transferred into their individual wells on the 96-well plates. Selected wells were either inoculated with 0 mM or 5 mM of DETA/NO·. Additionally, each well was inoculated with varying concentrations (e.g. 0, 20, 40, 60, 80, 160, and 320 ng/mL) of anhydrotetracycline (a less toxic derivative of tetracycline) to further induce expression from pRMC2. The growth curves were analyzed using a BioTek Synergy H1 microplate reader that tracked absorbance in 15 min intervals over a 24-hour period (Growth OD_{650}: 37°C, 97 kinetic cycles; First shaking was 830 seconds, orbital mode with a 1 nm amplitude. The second shaking consisted of a 30 sec duration, linear mode, 1 mm amplitude, and 10 second settle time). A total of three replicate growth curves with four or more sets of seven induction controls were conducted to determine and confirm bacterial growth relative to their expression of c-di-AMP under stressful conditions.

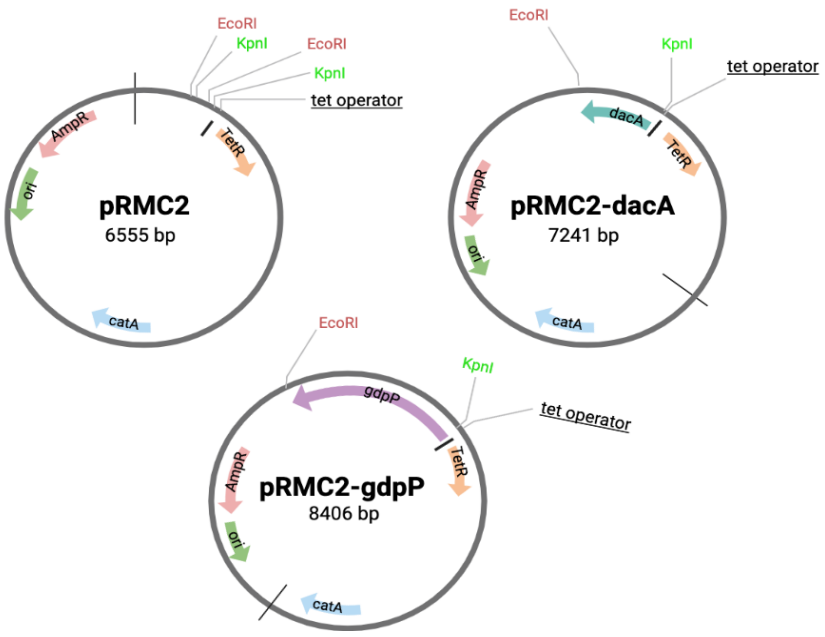

Figure 2. pRMC2 plasmids containing *dacA* and *gdpP* to allow us to control levels of c-di-AMP. pRMC2 is a plasmid for cloning a gene (e.g. *dacA* or *gdpP*) downstream of an inducible promoter. Then, the addition of anhydrotetracycline (aTc) can induce expression of the specific gene.

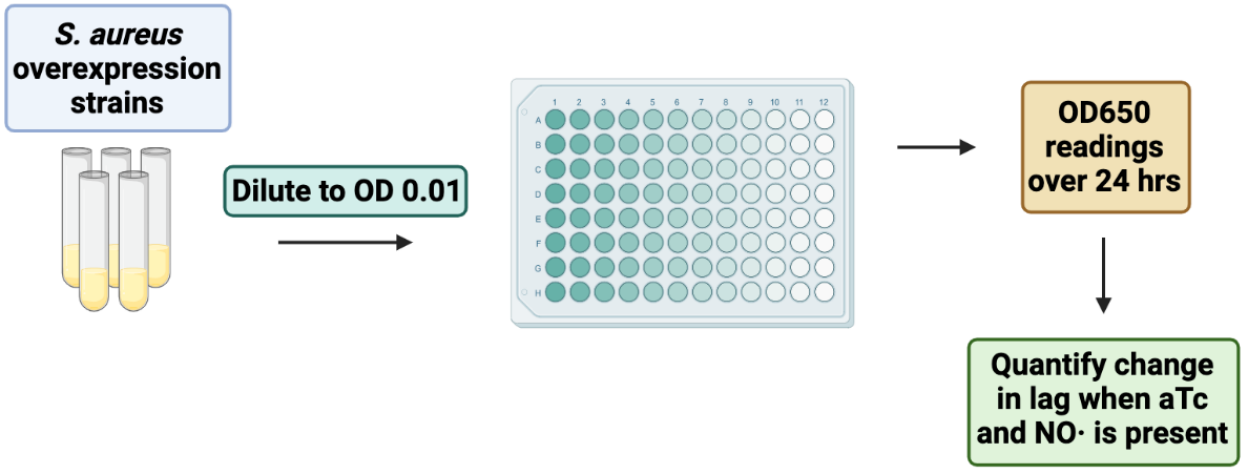

Figure 3. Schematic of assay for NO· sensitivity and aTc growth induction. *S. aureus* overexpression strains were grown overnight, diluted to an optical density (OD) of 0.01 and grown with different concentrations of aTc. In addition, the bacteria were grown in the absence or presence of DETANO/NO (an NO· donor).

2.2 Preparation of Buffers and Reagents for ELISA

To make the Immunoassay Buffer C (1x) required for the ELISA, one vial of the Immunoassay Buffer C (10x), provided by the kit, was diluted with 90 mL of DI water. When a smaller volume of buffer was

needed, a 1:10 dilution was calculated to create the buffer. It is important to note that the buffer may contain crystalline sales, which will dissolve upon dilution. The Wash Buffer (1x) was diluted by adding one vial of the Wash Buffer Concentrate (400X) and 1 mL of Polysorbate 20 to a total of 2 L of DI water. When a smaller volume was needed, a 1:400 dilution was made by adding 0.5 mL of Polysorbate 20 per every 1 L (e.g. 1.25 mL of Wash Buffer (400x), 500 mL of DI water, and 250 µl of Polysorbate 20). The consistency of Polysorbate 20 does not allow for proper measurement using a regular pipette tip. Therefore, the tip of a regular pipette tip was cut to allow for proper measurement. Lastly, to prepare a Tris buffer (50 mM Tris, pH 8, containing 20 µg of lysostaphin), weigh and add 2.22 g of Tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl) and 4.92 g of Tris B into a 500 mL container. Then, 500 mL of DI water was added into the container before it was mixed thoroughly. In a 15 mL conical tube, 10 mL of the Tris buffer was added into the tube. Next, 0.0002 g of lysostaphin was weighed and added into the 10 mL tube and mixed thoroughly before stored in the fridge at 4°C.

For the protein reader, a 1 mg/mL BSA Solution and Bradford Reagent was prepared**.** For the 1 mg/mL BSA solution, 10 mL of sterile water was measured into a 15 mL conical tube. Next, 10 mg or 0.01g of BSA powder was transferred into the conical tube and mixed thoroughly before stored at 4°C. For the Bradford reagent, 40 mL of sterile water was measured into a 50 mL conical tube. Then, 10 mL of Bradford reagent was added and mixed thoroughly before storage at 4°C.

2.3 Lysate Preparation for ELISA

Overnight cultures of *S. aureus* overexpression strains WT-LAC pRMC2 vector control, WT-LAC pRMC2-dacA, and WT-LAC pRMC2-gdpP strains were inoculated and grown overnight with with 5.0 mL of TSB, 5µL of 10 mg/L chloramphenicol antibiotics (Cm10), and 50 µL of aTc at a 1:10 dilution with a final concentration of 100 ng/mL. Then, the cultures were shaken and incubated at 37°C for 24 hrs. The following day, a 1:10 dilution with 900 µL of TSB and 100 µL of culture was made for each overexpression strain. Then, 500 µL of each diluted bacterial culture was transferred into three cuvettes to determine the OD at a wavelength of 650 nm; ideal OD values were 0.7. Following the OD reading, 5 mL of each culture was pipetted into a clean 50 mL conical tube and centrifuged for 5 min at 4°C and 5000 x g rpm. Pellets were washed and harvested with 1 mL of PBS, pH 8. Following the wash, cells were suspended in 1 mL of lysis buffer (50 mm Tris, pH 8, containing 20 µg of lysostaphin) and incubated at 37°C for 15 min. Lysates were added to a microcentrifuge tube with 0.5 mL glass beads and lysed using a Fast-Prep instrument at Setting 6 for 30 seconds; this procedure was repeated twice with 2 min on ice between runs. To remove the glass beads, samples were centrifuged at 17,000 rpm at 4°C for 5 min. Once the glass beads had settled to the bottom of the tube, the clear lysate was transferred to a fresh, sterile microcentrifuge tube before boiling it in a water bath for 10 minutes at 95° C. After the hot bath, lysates were centrifuged again for 1-2 min at 15,060 rpm to remove any additional debris. Finally, supernatants were moved into a new microcentrifuge tube and stored at 4°C or in an ice bath.

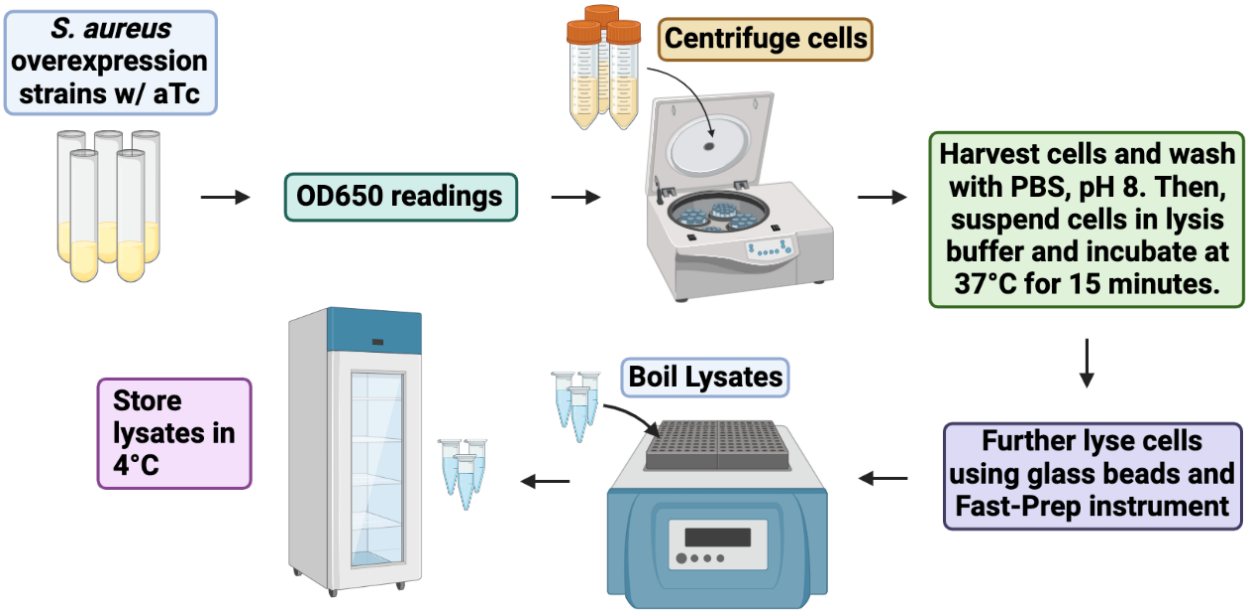

Figure 4. Schematic of culture preparation for ELISA to quantify c-di-AMP.

2.4 Total Protein Quantification of Lysates

Using a 96 well plate, 250 µL of Bradford reagent were added into the number of desired wells. For the standard wells, 0-5 µL of BSA solution was distributed into the first two columns (**Figure 5**). Following this step, 5 µL of the lysate samples were inoculated into their designated wells. Then, the plate was loaded into the BioTek Synergy H1 microplate reader for analysis of each individual well to determine the dilution needed to proceed with performing the ELISA. Each dilution was assayed in duplicate.

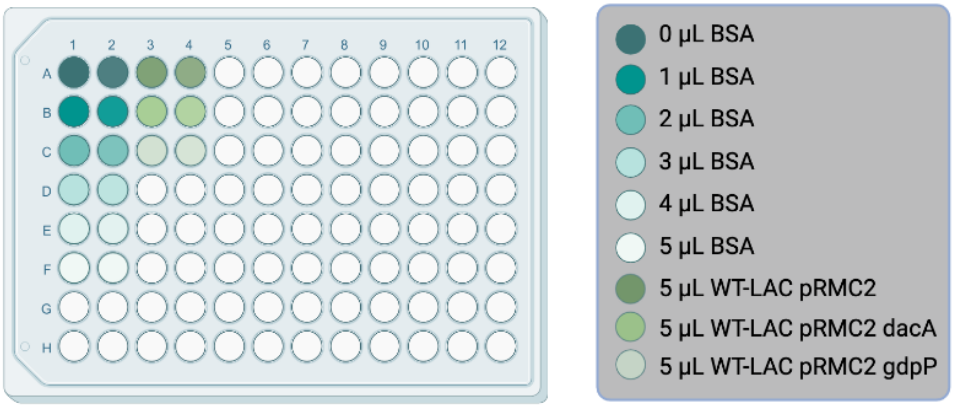

Figure 5. Example of Lysate Protein Quantification Set-up.
Before beginning the ELISA, quantification of proteins in each *S. aureus* overexpression strain lysates was crucial. Protein quantification allows us to determine the dilution factors for an effective measurement of c-di-AMP in samples during ELISA so the concentrations are not out of range. To all of the wells, 250 µL of Bradford reagent was added before the addition of BSA solution or lysate samples.

2.5 Preparation of Cyclic di-AMP ELISA Standards

To begin, 0.75 mL of Immunoassay buffer C (1X) was inoculated and mixed into the small container with the lyophilized (freeze-dry) cyclic di-AMP ELISA standard (provided by the kit) to create a 20 ng/mL bulk standard. This solution is proposed to be stable for at least four weeks when stored at 4°C. Next, with eight clean microcentrifuge tubes labeled #1-8, we conducted a serial dilution by adding 900 µL of the Immunoassay Buffer C (1X) to tube #1 and 500 µL of the Immunoassay Buffer C (1X) to tubes #2-8. Then, 100 µL of the bulk standard was transferred to tube #1 and mixed thoroughly. The standards were diluted following this step. From tube #1, 500 µL was transferred to tube #2 and mixed thoroughly. This step was repeated until all eight tubes were diluted.

2.6 ELISA Plate Set-up and Performing the ELISA

Prior to beginning the ELISA, 96-well plates provided by the ELISA kit were washed five times with ~300 µL of 1x Wash Buffer. As advised by the commercial ELISA user manual, a diagram of the plate set-up was created so that there would be a minimum of two blank (Blk), two non-specific binding (NSB), and two maximum binding (B_0) wells for an eight-point standard curve to run in duplicate. An example plate set-up is shown in **Figure 6**.

After finalizing the plate set-up, 100 µL of Immunoassay Buffer C (1X) was transferred to the NSB wells and 50 µL of Immunoassay Buffer C (1X) was transferred to the B_0 wells. Then, 50 µL of the Cyclic di-AMP ELISA standard from tube #8 was inoculated to both of the lowest standard wells labeled "S8". For all of the standard wells, this step was repeated for the "S1-S7" wells until all the standards were aliquoted. Next, 50 µL of bacterial samples (the supernatant that was collected after the lysis and heat bath) were distributed into their designated wells. Then, 50 µL of Cyclic di-AMP-HRP Tracer was added to each well except for the total activity (TA) and blank (Blk) wells and 50 µL of Cyclic di-AMP ELISA Monoclonal Antibody was added to each well except for the TA, Non-Specific Binding (NSB), and Blk wells within 15 minutes of adding the tracer. The plate was covered by a 96-Well Plate Cover Sheet provided by the Cayman c-di-AMP ELISA kit and incubated for 2 hours at room temperature. Afterwards, the wells were emptied and washed five times with the Wash Buffer (1X). We proceeded by adding 175 µL of TMB Substrate Solution to each well before 5 µL of the diluted tracer was transferred to the TA wells. Following this, the 96 wells plate was put through an orbital shaker at room temperature for 30 min with a plastic film. After 30 minutes, 75 µL of HRP Stop Solution was transferred to each well. Lastly, the plate was analyzed using the BioTek Synergy H1 microplate reader at a wavelength of 450 nm.

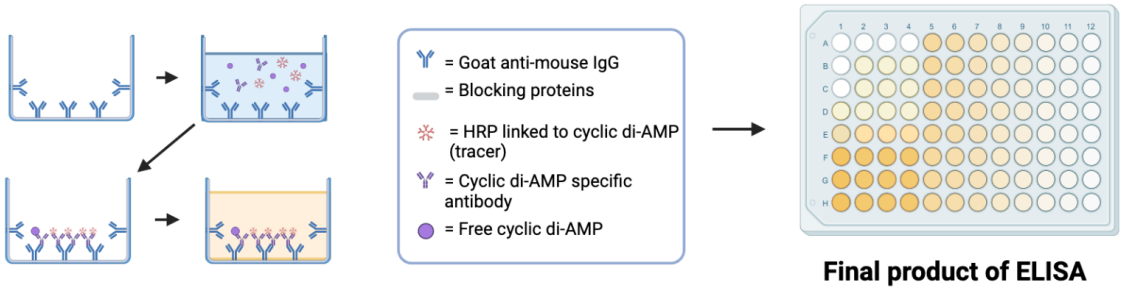

Figure 6. Schematic of Performing the Cayman Chemical Company Cyclic di-AMP ELISA. Tracer, antibody, and standards or samples were inoculated into each well before incubation. The wells were emptied and washed for 5x with the Wash Buffer (1X) after incubation. Next, the wells were developed with TMB substrate solution and HRP Stop solution before being analyzed in the BioTek plate reader at 450 nm. (Schematic adapted in BioRender based on Cayman Chemical Cyclic di-AMP ELISA user manual).

3. Results

3.1 *S. aureus* DacA overexpression strains, resulting in elevated c-di-AMP, cause toxicity when DETANO/NO· stress is present.

Aerobic and NO· growth curves were performed on *S. aureus* WT pRMC2 vector control and WT pRMC2-dacA overexpression strains induced by varying concentrations of aTc. In our assay, we found that when DacA overexpression strains are exposed to 5 mM NO· at the highest aTc concentration, DacA expression is induced to a level that is toxic for cells. During normal aerobic growth, increasing aTc concentration in the DacA overexpression strain has minimal effect on bacterial growth, whereas during NO· stress growth is impacted even at lower levels of DacA overexpression **(Figure 7, left panel).** For statistical analysis, we quantified lag time, or the time the strain needed to reach exponential growth (defined as OD_{650} 0.2) under each condition on aerobic and NO· growth curves of *S. aureus* WT pRMC2 vector control and WT pRMC2-dacA overexpression strains induced by higher varying concentrations of aTc **(Figure 8)**. During aerobic growth, there is a longer lag time for the WT pRMC2-dacA strain relative to vector control at the highest tested aTc concentration of 320 ng/ml (****, $p<0.0001$). During NO· stress, this longer lag time for WT pRMC2-dacA occurs at a lower level (160 ng/ml) of aTc induction (**, $p<0.01$; ****, $p<0.0001$). Therefore, this demonstrates that the overproduction of c-di-AMP may eliminate the ability for *S. aureus* to grow during NO· stress, so maintaining proper regulation is essential for its survival especially during nitrosative stress.

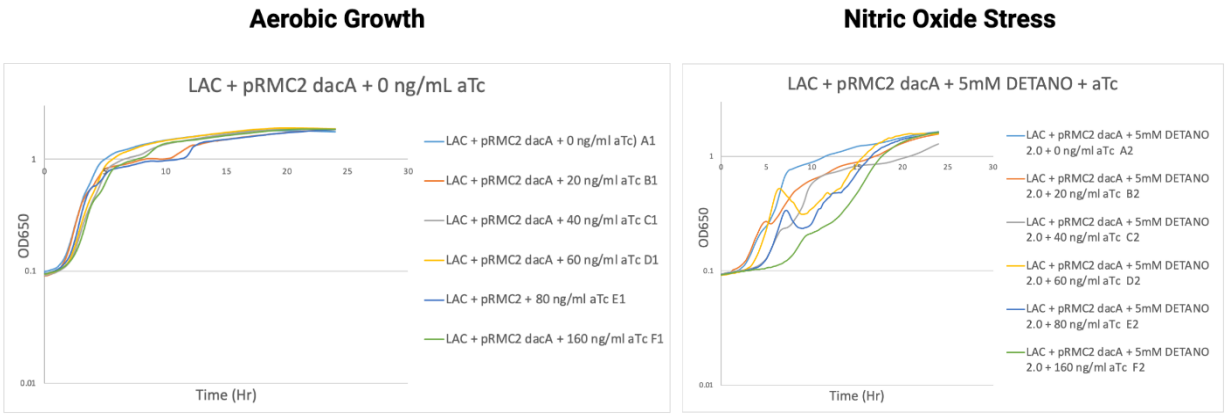

Figure 7. Growth Curves of *S. aureus* DacA overexpression strains (induced by aTc) in the presence and absence of DETANO/NO·. When DETANO/NO· is added to cause NO· stress (right panel) bacterial growth rates decline with increasing aTc concentrations, as DacA expression is induced to a level that is toxic for cells. Increasing aTc concentration has minimal effect during normal aerobic growth (left panel).

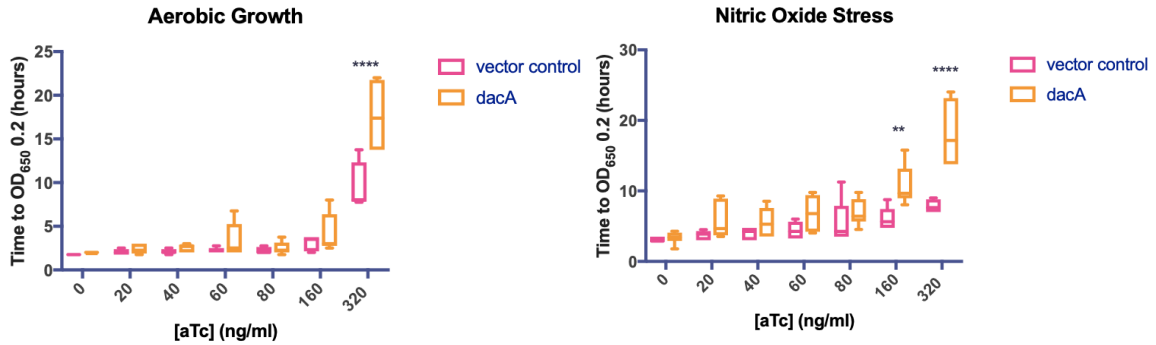

Figure 8. Average Time for WT pRMC2 (vector control) and WT pRMC2 dacA to reach exponential growth (OD_{650} 0.2) under aerobic growth (Without NO·) or in 5mM DETANO (With NO·). A two-way analysis of variance test (ANOVA) with a post hoc Bonferroni multiple comparison test revealed a significant difference in the time it took to reach exponential growth of WT pRMC2 vector control and WT pRMC2-dacA induced by varying concentrations of aTc under different growth conditions.

3.2 ELISA successfully quantified concentrations of c-di-AMP in *S. aureus*

Overnight cultures of *S. aureus* overexpression strains (WT-LAC gdpP, WT-LAC dacA, and WT-LAC pRMC2) were incubated with 50 µL of aTc at a 1:10 dilution to a final concentration of 100 ng/mL for 24 hours prior to performing the assay. The cultures were collected and diluted to a 1:10 final concentration to determine the OD reading for each strain. Then, we performed further lysate purification to quantify the lysates in a protein reading program on the BioTek Synergy H1 microplate reader. Then, the lysates were diluted to create a 1:2 and a 1:10 dilution to begin the assay. Following the analysis of our assay, ELISA confirmed that WT pRMC2-dacA overexpresses levels of c-di-AMP by ten-fold compared to WT

pRMC2 vector control and WT pRMC2-gdpP. In addition, WT pRMC2 expressed levels of c-di-AMP expected of WT, and WT pRMC2-gdpP expressed levels of c-di-AMP relatively lower than the pRMC2 vector control and pRMC2-dacA strains, although this was a smaller difference than expected. Therefore, further replication is needed.

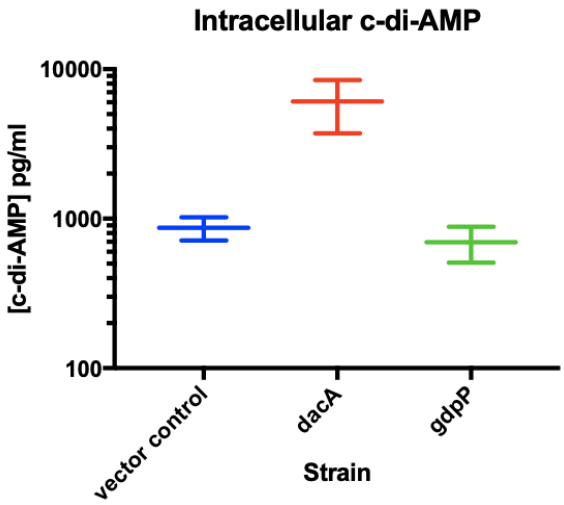

Figure 9. The lysis purification method yielded an acceptable concentration of proteins for use in ELISA. (n=2; further replication of ELISA is needed for statistical analysis).

4. Discussion

Staphylococcus aureus, an opportunistic pathogen, is infamous for its virulence in allowing it to successfully adapt and proliferate despite exposure to different stresses such as those imposed by the host immune system (Grosser et al. 2016; Cheung et al. 2021). The second messenger c-di-AMP is an important factor contributing to the pathogenesis of *S. aureus*. Here, we aimed to understand the significance for *S. aureus* to maintain optimal levels of c-di-AMP when subjected to nitrosative stress. We manipulated c-di-AMP levels by using a plasmid where dacA (respectively, an enzyme encoding the synthesis) is overexpressed under an inducible promoter. Based on the ELISA data, WT pRMC2-dacA produces more c-di-AMP than a vector control strain. In addition, when NO· is introduced, elevated c-di-AMP concentrations are especially toxic to *S. aureus* relative to during aerobic growth. This suggests, and is consistent with the data of Corrigan et al. 2011, that proper regulation in maintaining optimally low levels of c-di-AMP is beneficial for *S. aureus* to survive during nitrosative stress. Our data also suggest that the commercial ELISA used in this experiment, though originally designed for analysis on Gram-negative bacteria, is able to be modified and used for Gram-positive bacteria like *S. aureus*. Lastly, it is unclear if overexpression of WT pRMC2-gdpP is working. WT pRMC2-gdpP is producing a similar amount of c-di-AMP as WT, though it trends slightly lower (**Figure 9**). Therefore, further tests are needed to assess whether WT pRMC2-gdpP exhibits lower c-di-AMP levels.

Future work will be done on investigating the phenotypic overexpression characteristics of WT pRMC2-gdpP and why this strain is not exhibiting the expected degradation expression. We will repeat the ELISA with the same conditions from the NO· growth curves to determine the c-di-AMP levels in cells when growth defects are first observed. In addition, to investigate how levels of c-di-AMP normally fluctuate during NO· stress in live cells and real time, we will use the ELISA to calibrate a fluorescence resonance energy transfer (FRET)-based biosensor for c-di-AMP in *S. aureus* cells. Overall, our findings present a way to quantify intracellular c-di-AMP concentrations and may also provide some insight into the

importance of c-di-AMP signaling under nitrosative stress. This data could help identify potential antibiotics to disrupt the c-di-AMP signaling pathway in *S. aureus* to provide new therapeutic treatments.

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