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Exploring *dacA* expression knockdown during nitric oxide stress in *Staphylococcus aureus*

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Abstract

Staphylococcus aureus is the leading cause of skin and soft tissue infections in the United States and leads to diseases such as bacteremia, infective endocarditis, and pneumonia. Beta-lactam antibiotics have been the primary treatment since their first use in the 1940s; however, antibiotic overuse has led to the development of resistant strains such as methicillin-resistant *S. aureus* (MRSA). In addition to antibiotic resistance, MRSA has unique defenses against the host immune system; one example is its resistance to nitric oxide (NO·) produced by host phagocytes. The *S. aureus* response to NO· is complex and involves many regulatory pathways. The second messenger cyclic-di-AMP plays a major role in the NO· stress response, but the specific details remain unclear. C-di-AMP is produced through the condensation of two molecules of ATP by the diadenylate cyclase enzyme DacA. Our lab previously found that elevated levels of c-di-AMP, due to overexpression of *dacA*, cause a small growth defect during aerobic growth, but this defect worsens during NO· stress. Thus, having too much c-di-AMP is especially toxic during NO· stress. In this project, we aimed to investigate the consequences of depleting c-di-AMP levels during NO· stress. To reduce expression of the *dacA* gene, we created knockdown strains using CRISPR interference. We verified that *dacA* expression was reduced more than 10-fold in all knockdown strains via quantitative real-time PCR. When *dacA* knockdown strains were grown aerobically, they exhibited a major growth defect relative to wild-type. However, when exposed to NO· stress, this defect decreased relative to wild-type, suggesting that reduced c-di-AMP levels have a lesser impact during NO· stress. Future work will include RNA sequencing to provide insight into changes in gene expression associated with the mutation.

Introduction

Staphylococcus aureus is a Gram-positive bacterium and the leading cause of skin and soft tissue infections in the United States [1]. It is an opportunistic human pathogen primarily found in hospital settings, but more recently, some strains have spread, causing infection in the community [2]. These hospital and community-acquired strains are the leading cause of bacteremia, infective endocarditis, and pneumonia in the United States [3].

S. aureus has unique defenses against the host immune system. One example of resistance against innate immune effectors is nitric oxide (NO·) resistance. NO· is a reactive nitrogen species that can damage heme, iron-sulfur clusters, and DNA [1]. NO· disrupts respiration in *S. aureus*, resulting in the derepression of *ddh*, *ldh1*, and *ldh2*, which work together so *S. aureus* can maintain redox homeostasis through heterolactic fermentation [1]. *S. aureus* can also reduce damage from NO· through carbohydrate and glucose transporters and *ldh* [1].

Preliminary research conducted by Grosser lab found the second messenger cyclic-di-AMP plays a major role in NO· stress response with elevated levels being toxic to *S. aureus* [1;4]. Cyclic-di-AMP is produced through the condensation of two ATP molecules by the diadenylate cyclase enzyme DacA [2]. DacA is a membrane-bound enzyme that produces cyclic-di-AMP. In the cell, c-di-AMP is degraded by the phosphodiesterase GdpP into pApA (Figure 1).

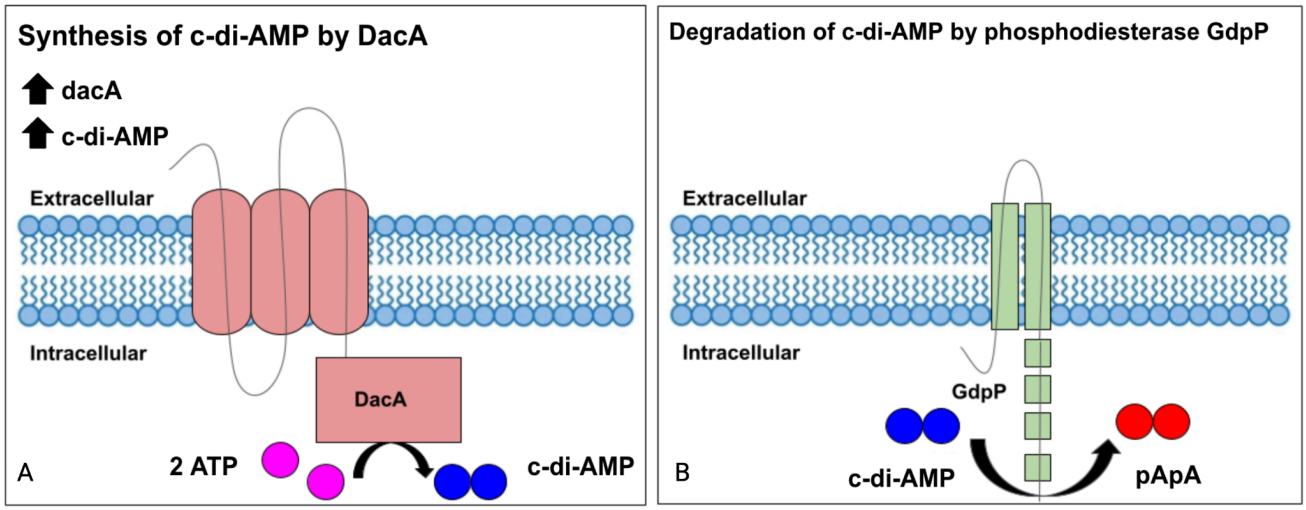


Figure 1. Depiction of c-di-AMP synthesis by DacA (panel A) and degradation by GdpP (panel B).

C-di-AMP is made in many species of Gram-positive bacteria, including *Streptococcus pyogenes*, *Listeria monocytogenes*, and *Mycobacterium tuberculosis* [5]. In *S. aureus,* c-di-AMP is involved in virulence, cell wall homeostasis, and resistance to beta-lactam antibiotics [6]. Elevated c-di-AMP levels have been shown to decrease cell size and increase cell wall thickness [4]. C-di-AMP regulates protein activity through direct protein binding in *S. aureus* [5]. Target proteins of c-di-AMP include the potassium transporter-gating component KtrA, part of the regulator of conductance of the K(RCK)

protein family, which is involved in ion channel gates [5]. Additional target proteins are CpaA, a cation and proton antiporter, KdpD, a histidine kinase, and PstA, a PII-like signal transduction protein [5]. All of these target proteins show the link between c-di-AMP and ion transport processes in *S. aureus*. C-di-AMP also inhibits and activates potassium systems and controls the gene expression of osmolyte transporters [6]. C-di-AMP is a second messenger that allows rapid adaptation to the host cell environment. For example, during osmotic changes in *S. aureus*, c-di-AMP regulates cell transport systems for potassium and osmolytes [2]. In *Listeria monocytogenes* and *S. aureus,* high levels of c-di-AMP have been linked to beta-lactam antibiotic resistance while low levels show less resistance [2].

S. aureus was primarily treated with beta-lactam antibiotics such as penicillin from the 1940s onward, with a decrease in their use since 1980 with the emergence of methicillin-resistant *S. aureus* (MRSA) [7]. Beta-lactam antibiotics work by binding to the transpeptidase domain of penicillin-binding proteins (PBP) and preventing them from cross-linking the polypeptide chains of the peptidoglycan cell wall [8]. Methicillin-resistant strains of *S. aureus* have the *mecA* gene, which encodes penicillin-binding protein 2a (PBP2a) and has a low affinity for beta-lactam antibiotics [8]. Another mechanism for resistance is expression of PC1 beta-lactamase which hydrolyzes the beta-lactam ring [9]. Mutations that elevate c-di-AMP levels represent a newly characterized and less understood mechanism of resistance. Due to antibiotic resistance, hospital-acquired and community-acquired *S. aureus* are challenging to treat.

Because c-di-AMP is connected to both immune and antibiotic resistance in *S. aureus*, *dacA* seems to be a promising candidate for research. The *dacA* gene is essential in *S. aureus*, meaning cells are not viable without it. Therefore, a deletion mutant cannot be used to study *dacA* function. Instead, we used Clustered Regularly Interspaced Short Palindromic Repeats interference (CRISPRi) to create knockdown strains. In these *dacA* knockdown strains, c-di-AMP levels should be decreased, allowing study into how the cell behaves when c-di-AMP is depleted during stress responses. CRISPRi uses the gene *dcas9* to encode a catalytically inactive Cas9 protein, which forms a complex with a single guide RNA (sgRNA) complementary to the *dacA* gene [10]. When the dcas9-sgRNA complex binds to the target DNA sequence, it sterically blocks initiation or halts RNA polymerase transcription elongation (Figure 2). This differs from the typical CRISPR/Cas9 system, where the targeted DNA would be cleaved in a double-stranded break, which would kill the cell since *S. aureus* lacks double-strand DNA break repair [1;4].

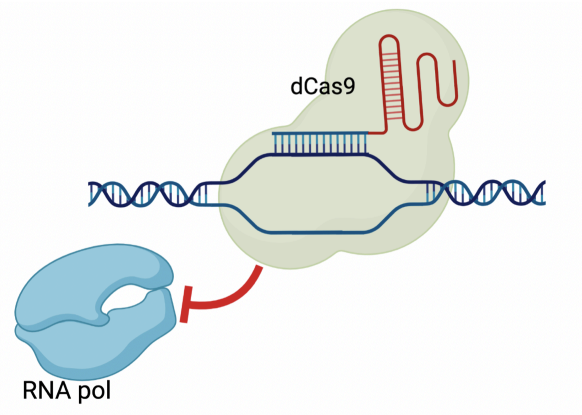


Figure 2. Depiction of CRISPR interference where *dCas9* binds to the DNA, physically blocking RNA polymerase from transcription.

This research project aimed to investigate the ability of *S. aureus* to overcome immune effector and antibiotic stress when c-di-AMP levels are reduced. This was done through the creation of three underexpression strains through CRISPR interference and then testing these strains in addition to overexpression strains against nitric oxide and kanamycin stress.

Materials and Methods

2.1 Strains and Culture Conditions

Overnight cultures of *S. aureus* strains were grown in tryptic soy broth (TSB) for 15 hours at 37°C while shaking at 250 rpm with 10μg/mL chloramphenicol added to ensure selective plasmid growth. The wild-type strain of *S. aureus* used in this research is USA300, a community-associated MRSA strain first identified in 1999 from an outbreak in a Los Angeles County prison [11]. USA300 is the second most commonly isolated strain type after USA100 and primarily colonizes the nasal passages [11]. In this research, USA300 is referred to as LAC for its place of origin (Los Angeles County).

The primers for creating the CRISPRi knockdown strains were created by students in Molecular Biology in Fall 2021. Students designed sgRNA for the *dacA* gene (Table 1), performed a ligation, and upon successful plasmid creation, transformed it into *E. coli*. Students in the course did not finish making the strains in *S. aureus*, so the final knockdown strains were completed as part of this project. In June 2024 I continued work on the strains from where the molecular biology students left off and transformed them using electroporation into two strains of *S. aureus*: RN4220 and, subsequently, LAC [12]. Plasmids are transformed into RN4220 first because it has an impaired ability to reject foreign DNA, which allows plasmids originating from *E. coli* to be uptaken and methylated so they can be transformed into LAC [4]. Because three different strains are used in the plasmid creation process, antibiotic markers were incorporated to confirm correct plasmid uptake. An ampicillin marker is used for *E. coli* and a chloramphenicol marker is used for RN4220 and LAC as they are both strains of *S. aureus* (Figure 3). In the CRISPRi plasmid, expression of *dCas9* and the sgRNA is repressed by TetR. Expression can be induced by the addition of anhydrotetracycline (aTc), which results in knockdown of the target gene.

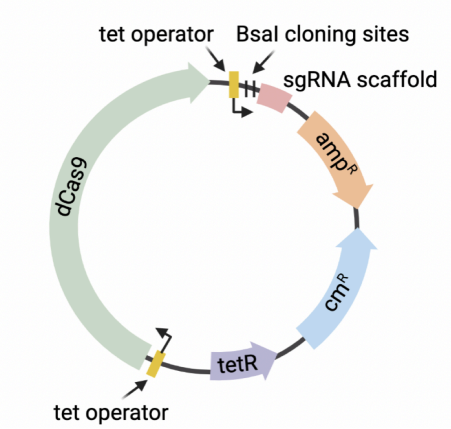


Figure 3. Plasmid map of the CRISPRi vector showing BsaI sites for cloning in the sgRNA, specially designed sgRNA, ampicillin marker to confirm uptake in *E. coli*, chloramphenicol marker to confirm uptake in *S. aureus*, and TetR which repressed *dCas9* until aTc is added so we have control over the expression of the gene of interest.

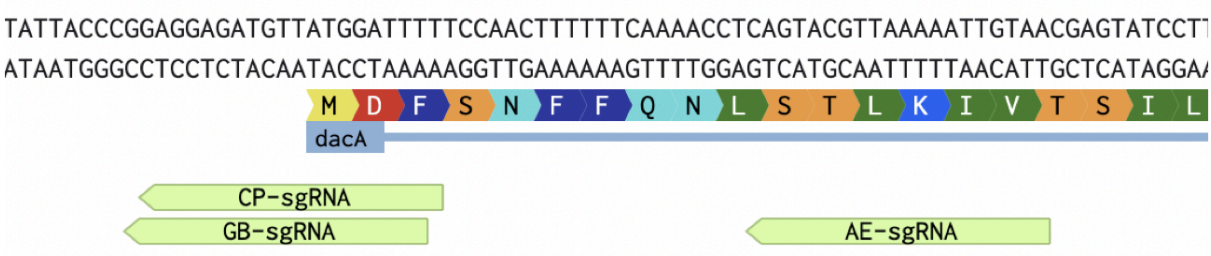


Figure 4. Map of sgRNA binding sites on the *dacA* gene with the N-terminal amino sequence shown above for the three CRISPRi knockdown strains that were created. CP and GB bind at the promoter, potentially blocking transcription initiation, while AE binds further downstream, potentially blocking elongation.

Some experiments also used a previously constructed LAC *dacA* overexpression strain (LAC pRMC2+dacA) where *dacA* expression is driven by an anhydrotetracycline-inducible promoter. LAC pRMC2 (empty vector) was used as a control strain for these experiments.

2.2 NO· and Kanamycin Growth Curve Assays

Overnight cultures were diluted 1:10 using TSB and the optical density at 650 nm (OD650) was measured using a Spectronic Genesys 2 spectrophotometer. Dilutions at an optical density of 0.01 were then created, and 200uL of each dilution was added to wells of a 96-well polystyrene plate. The plate was incubated in a Biotek Synergy HTX plate reader, while shaking at 37°C, using a standard growth curve procedure where OD650 was quantified every 15 minutes for 24 hours.

The first set of conditions had the three CRISPRi strains plus the control grown with nothing added, aTc at 400ug/mL, or aTc at 400ug/mL and 10mM (Z)-1-[N-(2-aminoethyl)-N-(2-ammonioethyl)amino]diazen-1-ium-1,2-diolate (DETANO), a NO· donor. Data were analyzed using RStudio.

The second set of growth curves included one CRISPRi strain, LAC GB, plus the control, and the overexpression strain pRMC2+dacA, plus its control, pRMC2. Kanamycin was added in a two-fold serial dilution with concentrations set at 0ug/mL,

1.5ug/mL, 3.125ug/mL, 6.25ug/mL, 12.5ug/mL, and 25ug/mL. All wells had aTc added at 200ug/mL to induce. Data were analyzed using RStudio. The minimum inhibitory concentration (MIC) was calculated by determining the lowest concentration where absorbance at 650nm did not exceed OD 0.15 at 14 hours.

2.3 Reverse Transcription Polymerase Chain Reaction (RT-PCR)

Three CRISPRi knockdown strains, the CRISPRi vector control, pRMC2, and pRMC2+dacA were grown with TSB and 10µg/mL chloramphenicol for 15 hours. The overnight culture was then diluted into 25mL fresh TSB along with 200 ug/mL aTc and 10µg/mL chloramphenicol and grown for 3 to 4 hours until an OD of 0.4 to 0.8 was reached. The 25mL culture was then combined with 25mL of a 50/50 mixture of acetone and 95% alcohol and frozen for 15 hours at -80°C to quench transcription.

RNA was then extracted and purified using a Qiagen RNeasy kit. Samples were treated with TURBO DNase to remove DNA from the purified nucleic acid mixture. The RNA was diluted to 10ng/mL and quantified using Applied Biosystems Onestep Plus instrument with SYBR-Green as the fluorescent dye to detect RNA. Three separate reactions were set up for each strain. The first did not include the reverse transcriptase enzyme to confirm that there was no DNA contamination in the sample. The second used primers to amplify the housekeeping gene *rpoD* that we normalized to and the third used primers to amplify our gene of interest, *dacA* (Table 1). Fold change was calculated through double delta Ct analysis. The cycle threshold (Ct) value is the number of cycles needed for enough RNA to be detected. The Ct values were averaged from the three replicates of each day to get values for the gene being tested experimental (TE), the gene being tested control (TC), the housekeeping gene experimental (HE), and the housekeeping gene control (HC). The difference between the experimental (TE - HE) and control values (TC - HC) were calculated to get delta Ct values for experimental (ΔCTE) and control (ΔCTC). The difference between the experimental and control ΔCt values gives the double delta Ct, which is in log base two. Taking the inverse of this log base gives the expression fold change with values above one indicating upregulation and values under one indicating deregulation of the gene of interest relative to the control.

Table 1. Primers used for RT-PCR and sgRNA primers for CRISPRi plasmids.

Primer	Sequence
rpoD	AACTGAATCCAAGTGATCTTAGTG
rpoD	TCATCACCTTGTTCAATACGTTTG
dacA	CCAACCAGAAATTAGACGTGC
dacA	TATATTGCACAGCCTTTGAAACC
GB sgRNA	AGCTCAAATCCATAACATCTCCTCCG

GB sgRNA	AAAACGGAGGAGATGTTATGGATTTG
CP sgRNA	AGCTCAAAATCCATAACATCTCCTCG
CP sgRNA	AAAACGAGGAGATGTTATGGATTTTG
AE sgRNA	AGCTCTTACAATTTTTAACGTACTGG
AE sgRNA	AAAACCAGTACGTTAAAAATTGTAAG

Results

Our first goal was to confirm whether the *dacA* CRISPRi knockdown and overexpression strains exhibited decreased or increased (respectively) expression of *dacA.* To investigate this, we extracted RNA from each strain after inducing plasmid expression with aTc. The RNA was then quantified using quantitative RT-PCR. Each of the three CRISPRi knockdown strains (LAC CP, LAC AE, and LAC GB) were found to have at least a ten-fold decrease in *dacA* RNA, normalized to the housekeeping gene *rpoD*, relative to the vector control strain. The overexpression strain (LAC pRMC2+dacA) had more than a one-million-fold increase, normalized to the housekeeping gene *rpoD*, relative to the vector control (Figure 5).

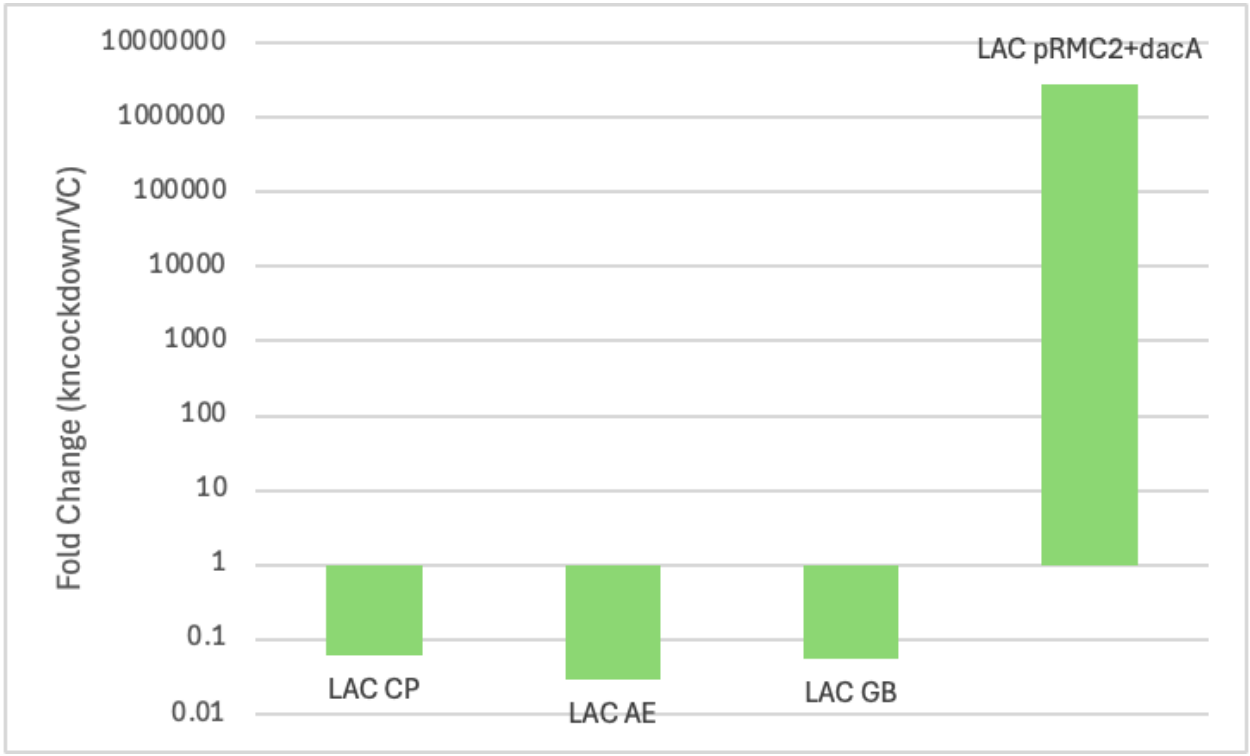
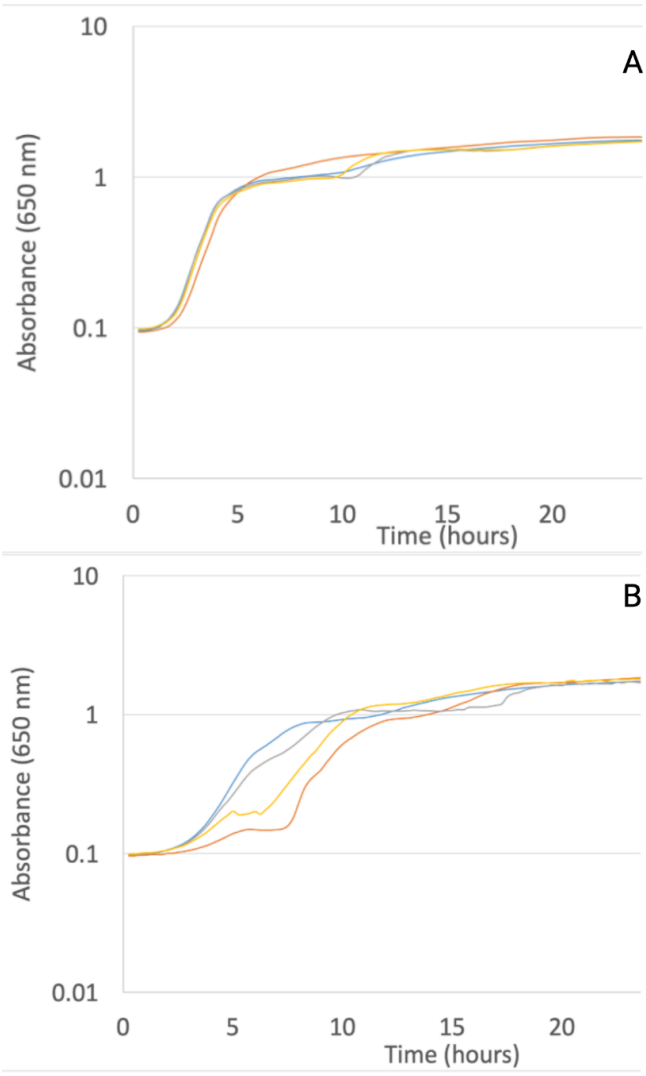


Figure 5. Representation of fold change after double delta Ct calculations for three CRISPRi strains (LAC CP, LAC AE, LAC GB) and one overexpression strain (LAC pRMC2+dacA)

normalized to the housekeeping gene *rpoD* and relative to their respective vector controls. Biological replicates varied from one to three on different days.

Our next goal was to test the ability of *S. aureus* to overcome immune effector nitric oxide stress when *dacA* expression was repressed. To examine if growth differences occur in *dacA* knockdown strains relative to vector control, a growth curve assay was performed. The bacteria were grown in TSB alone, induced with aTc, or grown with aTc and 10 mM of NO· for 24 hours. Previous research conducted by Taylor Gardner found when *dacA* overexpression strains were grown with NO·, *dacA* overexpression was significantly more toxic during NO· stress than during regular growth. In contrast, we found that *dacA* knockdown causes a major growth defect relative to the vector control during regular growth for the two strains with interference at the promoter. However, relative to the vector control, this defect does not worsen during NO· stress. LAC AE shows less of a growth defect when compared to LAC CP and LAC GB (Figure 6).



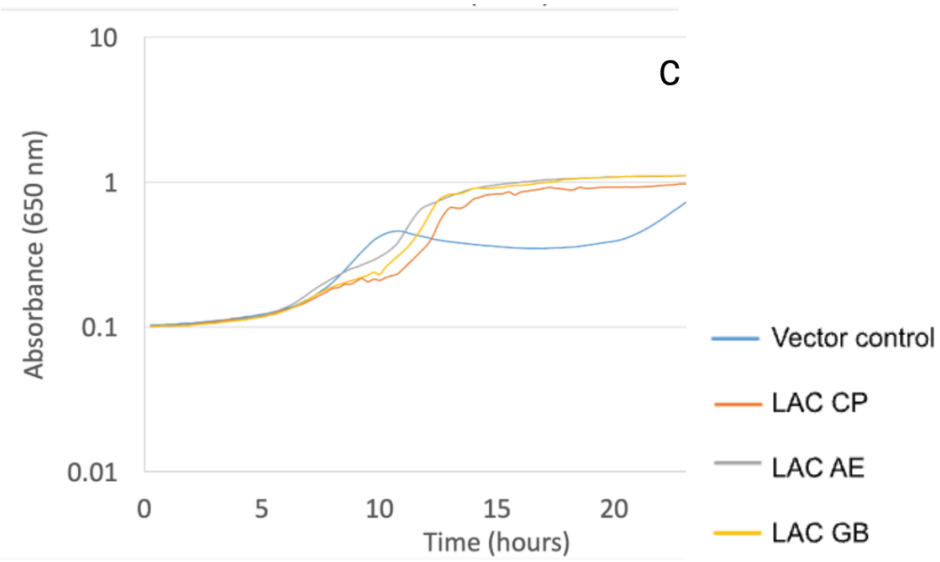


Figure 6. Representative growth curves of three CRISPRi strains and their vector control under normal conditions (panel A), aerobic conditions induced with aTc (panel B), and aerobic conditions induced with aTc and NO· stress (panel C). Three biological replicates on separate days were performed for each strain.

To quantify the difference in lagtime, time to reach OD 0.2 was calculated for each strain (Figure 7). A two-way ANOVA was done, which found a significant difference in each strain's growth between aerobic conditions induced with aTc and growth under nitric oxide stress ($F_{3,75}$=20.53, $p<0.001$). In both aerobic and NO· stress conditions, the vector control (LAC VC) and LAC AE grew similarly to each other. The same can be seen in LAC CP and LAC GB (Figure 7). LAC CP and LAC GB grew worse within aerobic and NO· stress compared to LAC AE and the vector control (Figure 7).

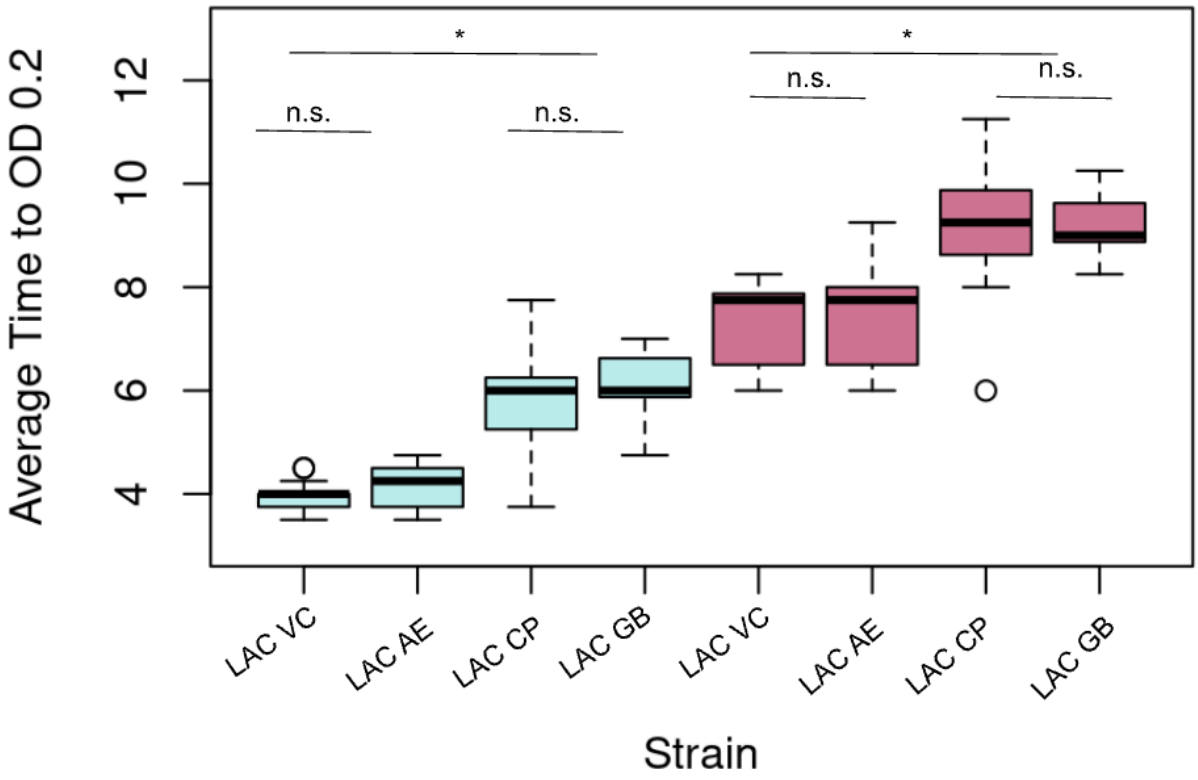


Figure 7. Average time to OD 0.2 for three *dacA* CRISPRi knockdown strains and vector control grown under aerobic conditions with 400 ug/mL aTc (blue) and under NO· stress with 400 ug/mL aTc and 10 mM DETANO (violet-red). Relative to the vector control, the *dacA* knockdown strain LAC AE performed similarly to the vector control while LAC CP and LAC GB performed similarly to one another. A two-way ANOVA found a significant difference in growth between the same strains when grown aerobically induced with aTc and grown under NO· stress ($F_{3,75}$=20.53, p<0.001). Three biological replicates on separate days were performed for each strain.

We wanted to see if *dacA* was more important in NO· stress than normal growth. To do this, the equation $(tNO\cdot - aerobic)_{knockdown}/(tNO\cdot - aerobic)_{WT}$ was used where aerobic lagtime was subtracted from NO· for both the knockdown strain and wildtype and then dividing the knockdown strain by the wild-type strain. If the resulting number was larger than one, then *dacA* was more important in NO· stress than normal growth, if lower than one, *dacA* was less important during NO· stress than normal growth. For all three CRISPRi strains (LAC CP, LAC AE, LAC GB), it was found that *dacA* was less important in NO· stress than in normal growth (Figure 8).

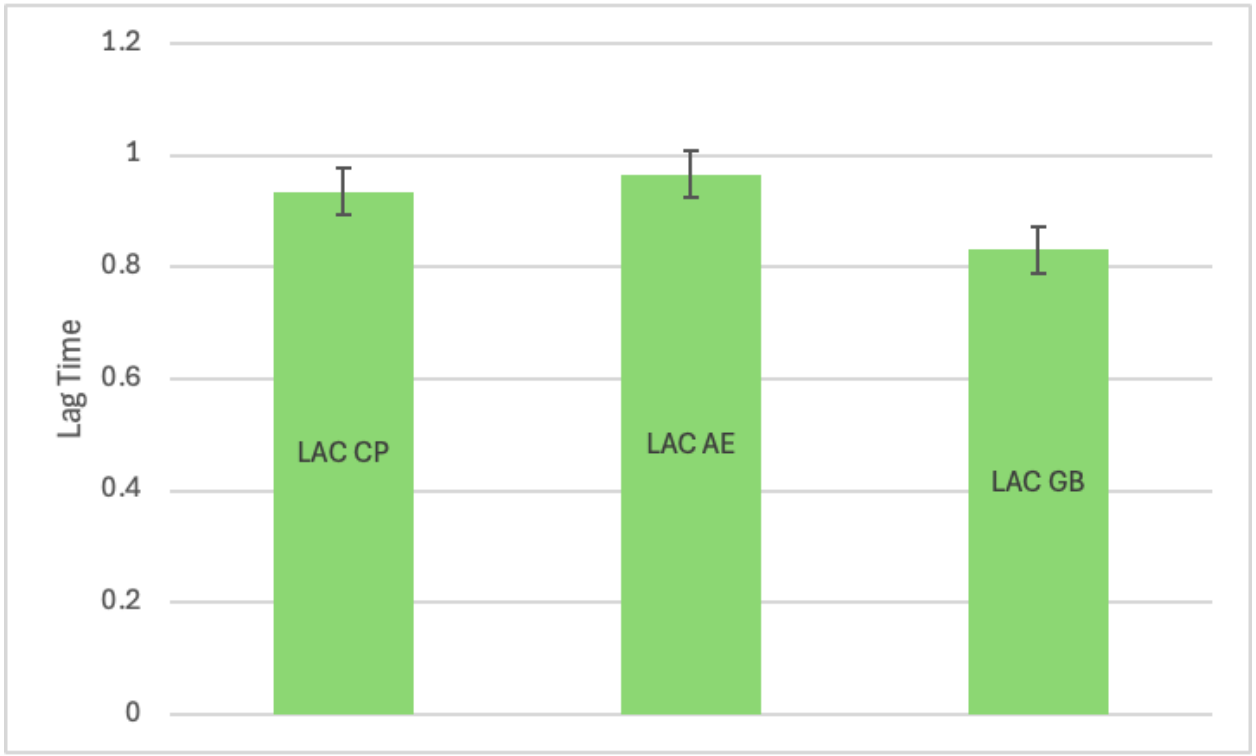


Figure 8. Quantification of *dacA* importance in NO· versus aerobic growth using the equation $(tNO\cdot - aerobic)_{knockdown}/(tNO\cdot - aerobic)_{WT}$. Calculations were performed on data collected from three biological replicates.

The next stressor studied was kanamycin, an aminoglycoside antibiotic that binds to the 30S subunit of bacterial ribosomes and inhibits mRNA translation into protein [13]. Previous literature has established that altered c-di-AMP levels impact beta-lactam resistance, so we were interested to see how they impact susceptibility to other classes of antibiotics with different cellular targets. A representative knockdown strain (LAC GB) and overexpression strain (LAC pRMC2+dacA) with their respective vector controls were used to determine the role *dacA* has when *S. aureus* is exposed to an aminoglycoside antibiotic. Growth curves were performed with the four strains grown in the presence of increasing concentrations of kanamycin (using 2-fold serial dilutions of the antibiotic). After growth, the minimum inhibitory concentration (MIC), the lowest concentration of kanamycin that inhibited growth for at least 14 hours, was calculated. Interestingly, there was no significant difference in MIC for the knockdown and overexpression strains relative to their vector controls (Figure 9), suggesting that altered c-di-AMP levels do not impact aminoglycoside sensitivity.

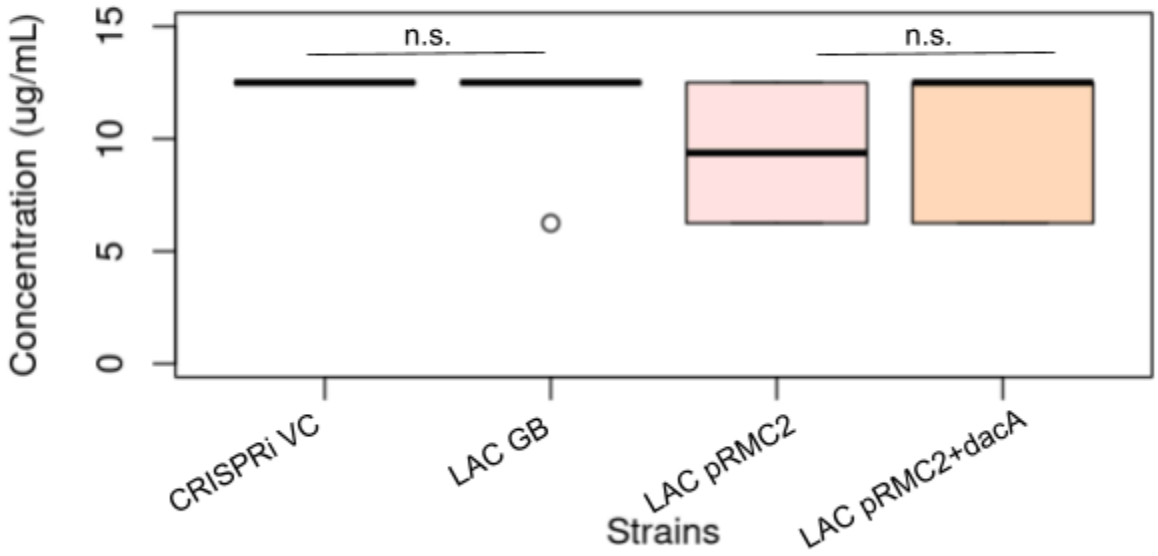


Figure 9. Boxplot of Kanamycin MIC data for knockdown and overexpression strains. There was a significant difference between the MIC of the two vector controls but no difference between the knockdown and overexpression strains and their respective vector controls ($F_{3,62}$=4.58, p=0.005). Three biological replicates on separate days were performed for each strain.

Discussion

In this research we demonstrate that *dacA* may serve an important role in *S. aureus* fitness during nitric oxide stress. We examined how the growth rate of *S. aureus* was affected in both overexpression and knockdown strains when exposed to nitric oxide and kanamycin stress. Previous results indicated that too much c-di-AMP negatively affects growth with NO·. Here, we found that reduced c-di-AMP levels caused a growth defect between knockdown strains and the vector control in both aerobic and NO· conditions.

In both the knockdown and overexpression strains, the precise levels of c-di-AMP need to be quantified. Based on the results of the RT-PCR we expect them to be decreased for the knockdown strains and increased for the overexpression strains. Our lab has previously used an ELISA (Enzyme Linked Immunosorbent Assay) to quantify c-di-AMP levels, so this is an important next step. The knockdown strain AE showed similar levels of *dacA* expression knockdown relative to the other knockdown strains, but exhibited less of a growth defect phenotypically, suggesting that c-di-AMP levels may not be as reduced in this strain. LAC AE had sgRNA that targeted *dacA* slightly downstream of the start codon (in contrast to the other knockdowns, which targeted the promoter). This could allow a small section of RNA to be transcribed, and perhaps a truncated version of DacA to be produced. The full amino acid length of DacA

is 270 (810 nucleotides) with approximately 10 amino acids synthesized before RNA polymerase is prevented from elongating the chain. Alternatively, it may be that CRISPRi is less effective at blocking transcription elongation relative to initiation. Regardless, this strain experienced less of a growth defect relative to the two strains with transcription blocked at the promoter. Further research will quantify c-di-AMP levels to confirm whether LAC AE is decreasing c-di-AMP levels and explain the lesser growth defect.

Future directions will include the continuation of growth curve assays to analyze the effect of overexpression and knockdown of *dacA* in the cell under additional stress conditions. RNA-sequencing will also be done to determine changes in global gene expression associated with altered second messenger concentrations, especially during NO· and kanamycin stress. We would also like to target the *dacA* knockdown and overexpression strains with other antibiotics, such as Linezolid, an oxazolidinone that targets bacterial protein synthesis, and Telavancin, a lipoglycopeptide that inhibits peptidoglycan synthesis and causes membrane depolarization [14;15].

Acknowledgments

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References

1. Grosser MR, Weiss A, Shaw LN, Richardson AR. 2016. Regulatory requirements for Staphylococcus aureus nitric oxide resistance. *J Bacteriol*. 198:2043–2055.

2. Tosi T, Hoshiga F, Millership C, Singh R, Eldrid C, Patin D, Mengin-Lecreulx D, Thalassinos K, Freemont P, Gründling A. 2019. Inhibition of the Staphylococcus aureus c-di-AMP cyclase DacA by direct interaction with the phosphoglucosamine mutase GlmM. *PLoS Pathog*. 15(1).

3. Tong SYC, Davis JS, Eichenberger E, Holland TL, Fowler VG Jr. 2015. Staphylococcus aureus infections: epidemiology, pathophysiology, clinical manifestations, and management. *Clin Microbiol Rev*. 28:603–661.

4. Grosser MR, Paluscio E, Thurlow LR, Dillon MM, Cooper VS, Kawula TH, Richardson AR. 2018. Genetic requirements for Staphylococcus aureus nitric oxide resistance and virulence. *PLoS Pathog*. 14(1).

5. Corrigan RM, Campeotto I, Jeganathan T, Roelofs KG, Lee VT, Gründling A. 2013. Systematic identification of conserved bacterial c-di-AMP receptor proteins. *Proc Natl Acad Sci USA*. 110:9084–9089.

6. Schwedt I, Wang M, Gibhardt J, Commichau FM. 2023. Cyclic di-AMP, a multifaceted regulator of central metabolism and osmolyte homeostasis in

Listeria monocytogenes. *Microlife*. 4(1).

7. Bush K, Bradford PA. 2016. B-lactams and β-lactamase inhibitors: An overview. *Cold Spring Harb Perspect Med*. 6(1).

8. Bæk KT, Gründling A, Mogensen RG, Thøgersen L, Petersen A, Paulander W, Frees D. 2014. β-Lactam resistance in methicillin-resistant Staphylococcus aureus USA300 is increased by inactivation of the ClpXP protease. *Antimicrob Agents Chemother*. 58:4593–4603.

9. Llarrull LI, Fisher JF, Mobashery S. 2009. Molecular basis and phenotype of methicillin resistance in Staphylococcus aureus and insights into new beta-lactams that meet the challenge. *Antimicrob Agents Chemother*. 53:4051–4063.

10. Reed P, Sorg M, Alwardt D, Serra L, Veiga H, Schäper S, Pinho MG. 2024. A CRISPRi-based genetic resource to study essential Staphylococcus aureus genes. *MBio*. 15(1).

11. Gao Y, Poudel S, Seif Y, Shen Z, Palsson BO. 2023. Elucidating the CodY regulon in Staphylococcus aureus USA300 substrains TCH1516 and LAC. *mSystems*. 8(1).

12. Grosser MR, Richardson AR. 2016. Method for Preparation and Electroporation of S. aureus and S. epidermidis. *Methods Mol Biol*. 1373:51–57.

13. John T, Thomas T, Abel B, Wood BR, Chalmers DK, Martin LL. 2017. How kanamycin A interacts with bacterial and mammalian mimetic membranes. *Biochim Biophys Acta Biomembr*. 1859:2242–2252.

14. Leach KL, Brickner SJ, Noe MC, Miller PF. 2011. Linezolid, the first oxazolidinone antibacterial agent. *Ann N Y Acad Sci*. 1222:49–54.

15. Saravolatz LD, Stein GE, Johnson LB. 2009. Telavancin: a novel lipoglycopeptide. *Clin Infect Dis*. 49:1908–1914.