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Investigating the role of the second messenger c-di-AMP during *Staphylococcus aureus* nitric oxide stress

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Abstract

Infectious diseases have emerged as the second leading cause of human death worldwide. *Staphylococcus aureus*, a Gram-positive bacterium, causes a variety of life threatening infectious diseases such as skin and soft tissue infections (SSTIs), osteomyelitis, pneumonia, and endocarditis. Moreover, *S. aureus* is unique compared to other bacterial species in that it is able to resist a variety of antibiotics and essential components of the host innate immune system, such as nitric oxide (NO·). In bacteria, NO· inhibits respiration and many metabolic pathways and can cause significant cellular damage, but *S. aureus* successfully adapts, detoxifies NO·, and alters its metabolism to allow for continued growth, unlike other bacterial species. *S. aureus* uses a second messenger called cyclic di-adenosine monophosphate (c-di-AMP) to regulate cell processes like stress response, cell wall size, and respiration. The small molecule c-di-AMP can function as an allosteric regulator of specific target proteins within the cell and is synthesized by the protein DacA and degraded by the phosphodiesterase enzyme GdpP. A previous transposon screen study *in vivo* on a community-acquired methicillin resistance *Staphylococcus aureus* strain (CA-MRSA) indicated that several genes associated with c-di-AMP signaling exhibited decreased fitness during NO· stress, including the gene SAUSA300_0730, encoding a hypothetical protein with a known second-messenger binding domain. However, it still is not well understood how c-di-AMP signaling relates to NO· stress. Here, we manipulated c-di-AMP levels by over-expressing the enzymes *dacA* and *gdpP* under an inducible promoter and found that high c-di-AMP levels are toxic during NO· stress. Furthermore, to examine the function of SAUSA300_0730 during NO· stress, site-directed mutagenesis was performed to create a deletion mutant, which exhibited slightly decreased fitness relative to wild-type. Altogether, our results suggest that proper regulation of c-di-AMP signaling is critical for the overall success of *S. aureus* under nitrosative stress. These findings may lead to identifying potential antibiotic targets to alter this signaling pathway.

1. Introduction

Staphylococcus aureus is a common bacterium known to cause major skin and soft tissue infections (SSTI), which have become more difficult to treat due to the pathogen's multi-drug resistant characteristics (Zeden et al., 2018). As the pathogen has gained virulence factors over time, it has become uniquely able to adapt to different stresses imposed by the host immune system (Grosser et al., 2016). In particular, *S. aureus* can grow in the presence of the host innate immune response radical nitric oxide (NO·). NO· is an important element of the innate immune response against bacterial infection, and is responsible for disrupting respiration, lipid biosynthesis, and other metabolic processes of bacterial cells (Grosser et al., 2018). NO· can exert various toxic effects on bacteria such as targeting metal centers of bacterial enzymes like iron-sulfur clusters and heme cofactors (Grosser et al., 2018). When NO· levels are high, the binding of NO· to metal centers inhibit respiration and other metabolic pathways, and unless the cell can adjust its metabolism, growth will be inhibited. In addition, NO· can react with oxygen to form a variety of toxic reactive

nitrogen species (RNS) and reactive oxygen species (ROS) that can target and damage bacterial DNA and lipids, leading to cell death unless the RNS and ROS are rapidly detoxified and damage is repaired (Grosser et al., 2016). In order to adapt and proliferate in the presence of NO·, *S. aureus* uses various signaling and metabolic processes that other species of bacteria either do not have or are not able to successfully activate in the presence of NO·.

To continue its pathogenesis, the ability of *S. aureus* to persist under NO· stress comes from a variety of intracellular mechanisms, but recent evidence suggests that some of these mechanisms may involve the second-messenger signaling molecule cyclic di-adenylate monophosphate (c-di-AMP). C-di-AMP has been discovered to help *S. aureus* and several other Gram-positive bacterial species like *Listeria monocytogenes* to adapt and cope under general stress conditions (Corrigan et. al., 2011; Zeden et al., 2018). This second messenger signaling molecule is synthesized from two ATP molecules by the protein DacA and is degraded into 5'-pApA by the phosphodiesterase enzyme GdpP (Figure 1), both of which are essential for the growth and viability of *S. aureus* (Corrigan et al., 2011). Second messengers can have a wide range of effects in bacterial cells, such as regulating simultaneously the activity of protein synthesis, transcription factors, and binding directly to mRNA to affect translation (Fahmi et al., 2017). For the well studied second-messenger cyclic di-GMP (c-di-GMP), the molecular mechanisms in bacterial cells are well understood by which it is able to regulate crucial cellular functions such as the transition from biofilm to motility (Corrigan et al., 2013). The ability of bacteria to transition from biofilm-motile form has been linked to colonization of the human host (Corrigan et al., 2013). However, currently there is no evidence for the existence of c-di-GMP in *S. aureus*, but rather it produces a similar second-messenger c-di-AMP. Current studies have found that c-di-AMP levels in *S. aureus* and other Gram-positive species are involved in regulating osmotic pressure, cell size and maintaining peptidoglycan synthesis, and transport of essential molecules such as potassium (K+) (Corrigan et al., 2013) (Figure 2). . Interestingly, when levels of c-di-AMP are too low, reactive oxygen species (ROS) toxicity is increased in *S. aureus*. Since ROS damage overlaps in many ways with NO· damage, this suggests that maintaining normal c-di-AMP levels during NO· stress may be essential (Corrigan et. al., 2011; Zeden et al., 2018).

A previous transposon sequence (Tn-seq) screen on a *S. aureus* community acquired methicillin resistance (CA-MRSA) strain identified a total of 168 genes that are likely to play a role in NO· resistance (Grosser et al., 2018). The genes SAUSA300_0730, *pstA*, and *ybbR* (Figure 1), which are all linked to c-di-AMP signaling, were deemed as important for fitness during NO· resistance (Grosser et al., 2018). More specifically this study indicated that transposon mutants in these genes exhibited decreased fitness relative to other transposon mutants in a competition assay during NO· stress (Grosser et al., 2018). SAUSA300_0730 has a GGDEF domain, typically involved in production of the second messenger c-di-GMP. Despite this, *S. aureus* does not produce c-di-GMP, so others have suggested that because this protein has binding sites for cyclic dinucleotides perhaps it has a role in c-di-AMP signaling instead. In addition, PstA has been discovered to directly bind to c-di-AMP and is therefore one of the mediators and targets of c-di-AMP signaling in *S. aureus*. Sequence homology suggests it is a P-II like signal transduction protein (Corrigan et al., 2011; Arcondéguy, 2001). The transposon experiment established PstA as being important for *S. aureus* during NO· stress (Grosser et al. 2018). However, the exact function and characterization of SAUSA300_0730 and PstA are still undetermined (Choi et al., 2017). Clean deletion mutants of SAUSA300_0730 or *pstA* have not yet been studied in the context of NO· stress or even confirmed to be sensitive to NO· when grown in pure culture, since the transposon screen looked only at the relative fitness of thousands of mutants in a pool (Grosser et al., 2018). Thus, to begin investigating if c-di-AMP in *S. aureus* is crucial during NO· resistance, this research aimed first to clarify the role of SAUSA300_0730 and *pstA* during NO· stress by constructing and examining growth properties of deletion mutants in each gene. Additionally, to more generally investigate the importance of cyclic-di-AMP levels during NO· stress, we constructed strains in which *dacA* or *gdpP* could be overexpressed from an inducible promoter to artificially manipulate cellular c-di-AMP levels.

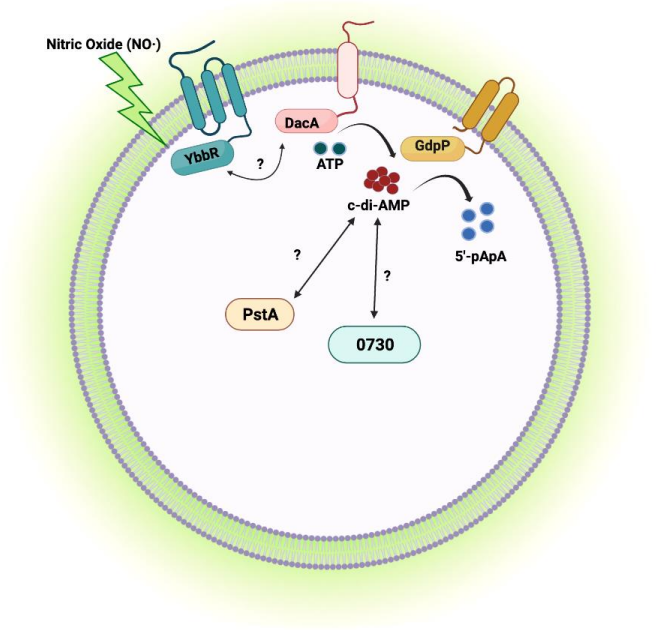


Figure 1. Signaling model of c-di-AMP synthesis and novel genes involved under nitrosative stress (shown in green) in *S. aureus*. DacA synthesizes c-di-AMP from two molecules of ATP (dark green circles) and GdpP degrades c-di-AMP into 5'-pApA. Ybbr, PstA and 0730 genes are associated with c-di-AMP signaling and are important under nitric oxide resistance, but the cellular functions of these genes under nitric oxide resistance are still not fully understood.

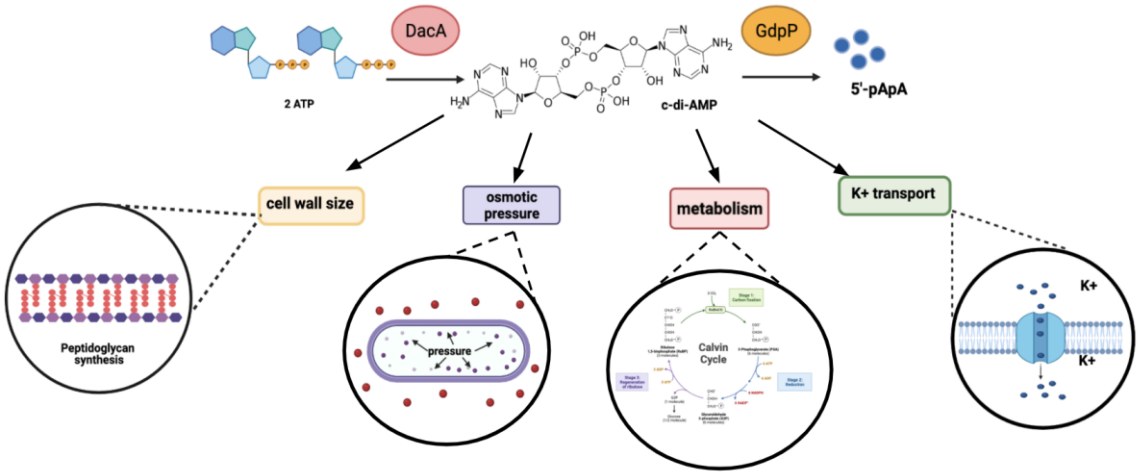


Figure 2. Model portraying a few of the important cellular processes that c-di-AMP is able to regulate based on previous studies in *S. aureus*. c-di-AMP can regulate cell processes such as cell wall size, osmotic pressure, metabolism and potassium (K+) transport.

2. Materials and Methods

2.1 Chromosomal Deletion Strategy and Manipulation of c-di-AMP levels

Allelic exchange was performed to create a chromosomal deletion in SAUSA300_0730 and *pstA* and replace each gene with an erythromycin resistance marker, *ermB*. Briefly, this was done by amplifying 1000 base pair flanking regions of each gene via polymerase chain reactions (PCR), cloning into the plasmid pBTE on each side of an erythromycin resistance gene to create a knock-out plasmid, transforming the KO plasmid into wild-type *S. aureus* cells and plating onto media containing erythromycin to select for mutants. Mutagenesis steps are described below in detail. To manipulate c-di-AMP levels, pRMC2 plasmids were constructed containing the overexpression strains dacA and gdpP and induced under an inducible promoter anhydrotetracycline (atet) and transformed into LAC for phenotypic comparison.

2.1.1 generation of knock-out plasmid for chromosomal deletion

To extract the pBTE plasmid from *E. coli*, an IBI High-Speed Plasmid Mini Kit was used and total DNA concentration (ng/ml) was measured using a BioTek plate reader. To purify *S. aureus* LAC gDNA, the Lucigen Gram Positive DNA purification kit was used. Polymerase chain reactions (PCR) using NEB Q5 High-Fidelity DNA Polymerase on the LAC genomic DNA template were performed to amplify the 1000 base pair flanking regions upstream and downstream of SAUSA300_0730 and PstA (SAUSA300_0460) using the primers listed on Table 1. A T100 thermal cycler was used for the PCR and the conditions were as follows: initial denaturation at 98°C for 30 seconds, 25-35 cycles at 98°C for 5-10 seconds, 50-72°C for 20-30 seconds, 72°C for 20-30 cycles per kilobase, a final extension at 72°C for 2 minutes, and held at 2-10°C. Gel electrophoresis of the PCR products confirmed the presence of successful amplification within the LAC gDNA. An IBI Gel/PCR DNA Fragments Extraction kit was used to purify the PCR products and DNA concentrations were quantified.

Table 1. List of primers used for this study

Primers	Sequence
USA300_0460-5’FWD	5’-CACTAGAATTCTGCTTCAAGTTGATGATCCG-3’
USA300_0460-5'REV	5’-CACTAGAATTCTTTATAACACCTCTTCTAAC-3’
USA300_0460-3'FWD	5’-CACTAGGATCCGCTTGACTGCGACTAGTTCA-3’
USA300_0460-3'REV	5’-CACTAGGATCCCAATTTCTTATGTGCTTCCG-3’
USA300_0730-5’FWD	5’-CACTAGGATCCAGGAATCCGATGATAAATCC-3’
USA300_0730-5’REV	5’-CACTAGGATCCATCGACTCCTTTAATTGACC-3’
USA300_0730-3’FWD	5’-CACTAGAATTCCAACGACAAAACTGTTAATCC-3’
USA300_0730-3’REV	5’-CACTAGAATTCGCGAATGCTGTTAATGCAGC-3’
gdpP-1	5’-CACTAGGTACCCTAAAAAGTGAATAGAG-3’

gdpP-2	5’-CACTAGAATTCCTTTCATGCATCTTCACTC-3’
dacA-1	5’-CACTAGGTACCTACCCGGAGGAGATGTTATG-3’
dacA-2	5’-CACTAGAATTCCATATTATTTCACACCTTTC-3’

2.1.2 cloning of 5’ and 3’ inserts of the chromosomal region flanking the target gene

To clone in the first insert (the 5’ chromosomal region flanking the target gene), the restriction enzyme BamHI was used to digest the 5’ flanking PCR product and the fragment was cloned into the BamHI site in pBTE for 5' flanking of 0730 (EcoRI for 5' flanking region of *pstA*). In order to prevent the plasmid restriction sites from self-annealing, the digested pBTE was treated with Antarctic phosphatase (NEB). Following this, a ligation was performed using the NEB standard T4 ligase protocol. The standard NEB Heat transformation protocol was done to transform ligated products into NEB DH 5-alpha High-Efficiency Chemically Competent *E. coli* cells. The cells were then plated onto media containing Lysogeny Broth (LB) agar + 100 µg/ml of ampicillin for single colony isolation. To confirm that the insert fragment was cloned in the right direction and present, One-Taq colony PCR was done. Confirmation of the directionality of the insert for each gene was performed using a plasmid-specific reverse primer (BT2.2b) purchased from Eurofins Genomics. Gel electrophoresis was then done to view successful colonies that were around a 1kb PCR product. These colonies contained the pBTE-0730-5’ and pBTE_*pstA*-3’, and were cultured into LB liquid culture with 100 µg/ml of ampicillin for antibiotic selection and shaken at 250 rpm at 37°C.. Following this, freezer stocks were made from the overnight cultures (O/N) using 50% glycerol and 500 µl of O/N culture and stored into a -80°C freezer.

In order to clone in the second insert (the 3’ chromosomal region flanking the target gene), the plasmid pBTE containing the 5’ flanking fragment was extracted from *E.coli* using the IBI High-Speed Plasmid Mini Kit. The 3’ flanking region of 0730 was cloned into the EcoR1 site within pBTE_*0730*-5’ from the same protocol listed earlier in the cloning of the 5’ insert. For *pstA*, the 5' flanking region was cloned into the BamHI site within pBTE_*pstA*-5’. For confirmation of the presence of the insert and correct orientation, One-Taq colony PCR was performed. BT2.2a, a plasmid-specific forward primer purchased from Eurofins Genomics, was used to confirm the orientation of the insert within pBTE for each gene. Following this, successful colonies were grown at 37°C overnight and shaken at 250 rpm in LB broth and 100 µg/ml ampicillin for antibiotic selection. Freezer stocks were made from the overnight cultures using 50% glycerol and 500 µl of O/N culture and stored at -80°C. To use the complete knock-out (KO) plasmid pBTE, a IBI High-Speed Plasmid Mini Kit was used to extract it to continue the development of each mutant.

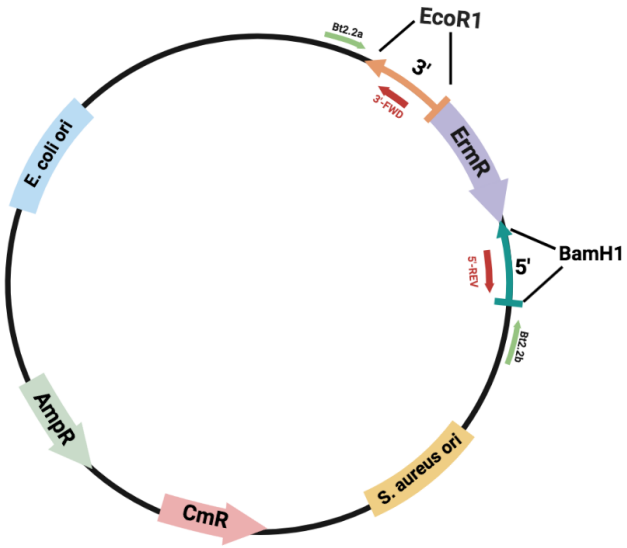


Figure 3. Knock-out plasmid containing the erythromycin resistance gene (ErmR), selection for ampicillin (AmpR) and chloramphenicol resistance (CmR).

2.1.3 creation of clean deletion of 0730 and psta in s. aureus *usa300 lac*

After the complete KO plasmid, pBTE_*0730* and pBTE_*pstA* were extracted and then transformed into *S. aureus* RN4220. This RN4220 strain of *S. aureus* is commonly used due to its restriction-deficient characteristics caused by a mutation in the *sau1 hsdR* gene (Nair et al., 2011), making it an ideal cloning host for the uptake of other *S. aureus* strains due to proper uptake of *E. coli* DNA and methylation. The RN4220 electrocompetent cells were prepared and electroporated as described in Grosser et al., 2018. Due to a temperature sensitive *S. aureus* origin of replication in pBTE, the product was plated and incubated at 30°C (the permissive temperature for plasmid replication) for 48 hrs. Following this, single colonies were grown overnight and shaken at 30°C in 5 µl of liquid tryptic soy broth (TSB) and 10 µg/ml chloramphenicol (Cm10) for antibiotic selection. The overnight cultures were then miniprepped using the standard IBI miniprep procedure with slight modification of the addition of 2500 µg/ml lysostaphin in order to lyse the *S. aureus* cell wall after PD1 resuspension. The DNA (ng/ml) was quantified using the BioTek plate reader.

The extracted pBTE KO plasmid was next ready for uptake by *S. aureus* due to proper methylation. The pBTE KO plasmid was transformed into *S. aureus* LAC electrocompetent cells which were prepared the same way as described above. The cells were then plated onto tryptic soy agar (TSA) media containing Cm10 and grown at the permissive temperature of 30°C. Single colonies were then isolated, shaken, and grown in TSB and chloramphenicol at a non-permissive temperature of 43°C to force homologous recombination of the plasmid into the chromosome of either *0730*-5' or 3' regions. Serial dilution plates were done to isolate colonies and the single colonies were grown in TSB media containing 5 µg/ml (Erm5) and no Cm10 and incubated at 30°C to allow for the generation of the chromosomal deletion of *0730*. This ensures that there would be a 50% chance of either generating a mutant or regenerating back into wild-type based on the recombination events. To ensure that the replicating cells were either mutants or wild-type, the presence of 5 µg/ml erythromycin (*ermB*) confirmed this. Following this, the plasmid was cured by a three-day passage of 30°C without Cm10.

Lastly, a cycloserine enrichment was performed for the isolation of mutant clones that had successfully lost the plasmid, making them Cm-sensitive. The overnight cultures were diluted 1:1000 into fresh TSB that did not contain any antibiotics and were incubated at 30°C. The new culture was then diluted 1:1000 in fresh TSB, shaken and grown at 37°C to an OD_{660} ~1.0 (~1 X 10^8 cfu/ml) before the addition of Cm10. Due to Chloramphenicol being a bacteriostatic antibiotic, it allows the growth of cells containing the plasmid to keep growing while also stopping the growth of any cells that lost the Cm-resistance containing plasmid. After a 30 minute incubation and shake, 1:100 (100 µg/ml) of D-cycloserine was added to the cultures. D-cycloserine is a bactericidal antibiotic that kills only growing cells, therefore this results in the preferential elimination of cells containing the Cm-resistant plasmid and thus enhancing for the cells that were able to lose the plasmid. The cultures were then plated onto serial dilutions with media containing TSA and

Erm5 and incubated at 37°C for 24 hrs. Single colonies were concurrently patched onto TSA + Erm5 and TSA + Cm10 plates for the detection of Cm-sensitive recombinants that would indicate the presence of a mutant. To confirm the mutant, a PCR was performed using Q5 High Fidelity DNA Polymerase and gel electrophoresis was done to visualize. Briefly, the PCR was done by using the primers BT2.2a, BT2.2b and a primer flanking the 5' and 3' chromosomal region of *0730* to confirm that the *0730* was replaced with the *ermB* cassette.

2.1.4 construction of pRMC2 plasmids

To create overexpression plasmids for *dacA* and *gdpP*, the plasmid pRMC2 was used. pRMC2 encodes the TetR repressor and has a multiple cloning site downstream of a tet operator. The complete genes for *dacA* and *gdpP*, plus ~14 bases upstream of the start codon to ensure the Shine-Delgarno was included for optimization of translation, were PCR-amplified using the primers listed in Table 1. These fragments were cloned into the KpnI/EcoRI sites of pRMC2 (Figure 6) and confirmed via PCR.

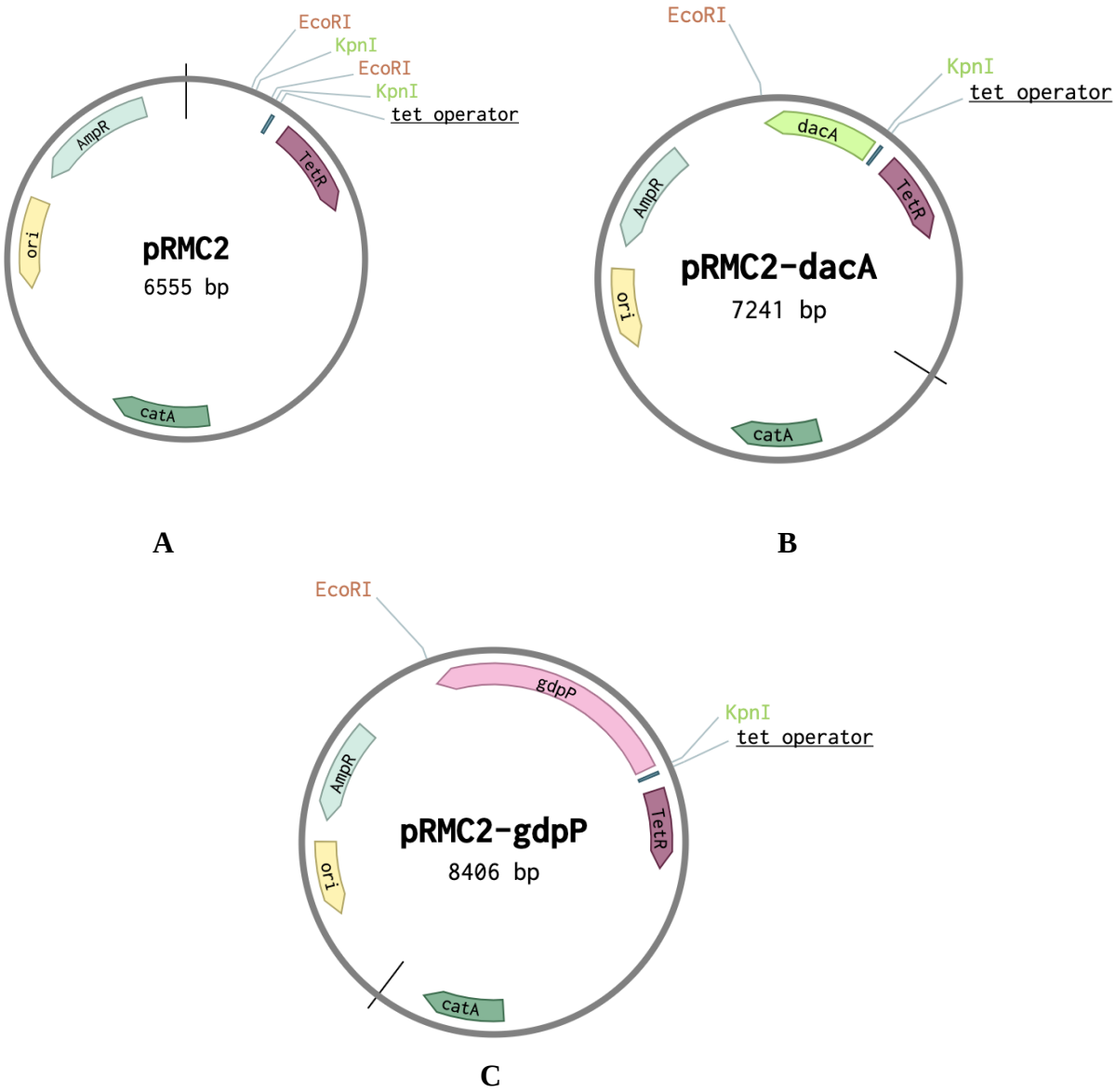


Figure 4. Constructed pRMC2 plasmids with a vector control (**A**), overexpression plasmid containing *dacA* (**B**) and *gdpP* (**C**).

2.1.5 transforming pRMC2 plasmids into LAC

The pRMC2, pRMC2-dacA, and pRMC2-gdpP plasmids were first cloned into *S. aureus* RN4220 because of it being restriction-deficient and the procedure was done exactly as described earlier for the KO plasmid, and grown on TSA + 10 µg/ml chloramphenicol (10 µg/ml) media for 24-48 hrs at 37°C. Successful colonies were grown and shaken overnight in liquid TSB containing chloramphenicol (Cm10) at 37°C. Freezer stocks were made and the cultures were mini-prepped to extract the plasmid and get it ready for the next cloning event. The pRMC2 plasmids were then transformed into *S. aureus* LAC electrocompetent cells and again plated on TSA + Cm10 at 37°C.

2.1.6 growth curve assays

Phenotypic growth comparisons were performed on the *Δ0730* mutant, pRMC2 vector control, pRMC2+dacA, pRMC2+gdpP relative to wild-type LAC. 24-hour growth curves were performed under a variety of conditions using 96-well plates and a BioTek plate reader was used to analyze the individual wells. The bacteria for the growth curves were prepared by growing each bacteria strain individually overnight with TSB (the LAC strains containing the pRMC2 plasmids were grown with Cm10 antibiotic added) and the cultures were shaken and incubated at 37°C for 24 hrs. Following this, each culture's OD was measured at 650 nm and diluted to a final OD_{650} of 0.01. 200µl of each sample was transferred onto individual wells on the 96-well plates. Selected wells were inoculated with 10 mM or 20 mM of DETA/NO·. Also, 40 nm anhydrotetracycline (a less toxic derivative of tetracycline) was used to induce expression from pRMC2. The growth curves were analyzed by the BioTek plate reader that tracked absorbance in 15 minute intervals (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, 10 second settle time, in 5 flashes).

3. Results

3.1 Generation of clean deletion mutant, *Δ0730*

Targeted mutagenesis was done to create the chromosomal deletion mutant, *Δ0730*, and its phenotype was characterized under NO· stress. First, a knockout plasmid (pBTE) from *E. coli* was constructed that consisted of an erythromycin resistance gene (*ermB*) that was flanked by DNA sequences of *S. aureus* USA300_0730. In *E. coli*, the complete KO plasmid (Figure 3) was extracted, purified and electroporated in the cloning host, the restriction-deficient RN4220 strain of *S. aureus* to allow for the uptake of *S. aureus* DNA. Subsequently, it was transformed into *S. aureus* LAC electrocompetent cells and grown under non-permissive temperatures to allow for homologous recombination event to occur between the flanking regions of *0730* and the plasmid that yielded first in a single recombination event of the integration of the entire KO plasmid into the *S. aureus* chromosome, and a second recombination event that yielded the removal of the KO plasmid from the chromosome. These events ensured the replacement of the chromosomal *0730* gene with the *ermB* gene within the plasmid. The chloramphenicol-resistant plasmid was cured through serial passages and enriched for colonies with chloramphenicol sensitivity and erythromycin-resistance. Colonies that grew on media containing Erm5 and ones that did not grow on media containing chloramphenicol were considered to be mutants (Figure 5). For further confirmation, the colonies were screened using Q5 PCR and this confirmed the replacement of *0730* with the *ermB* cassette and the loss of the KO plasmid (Figure 6).

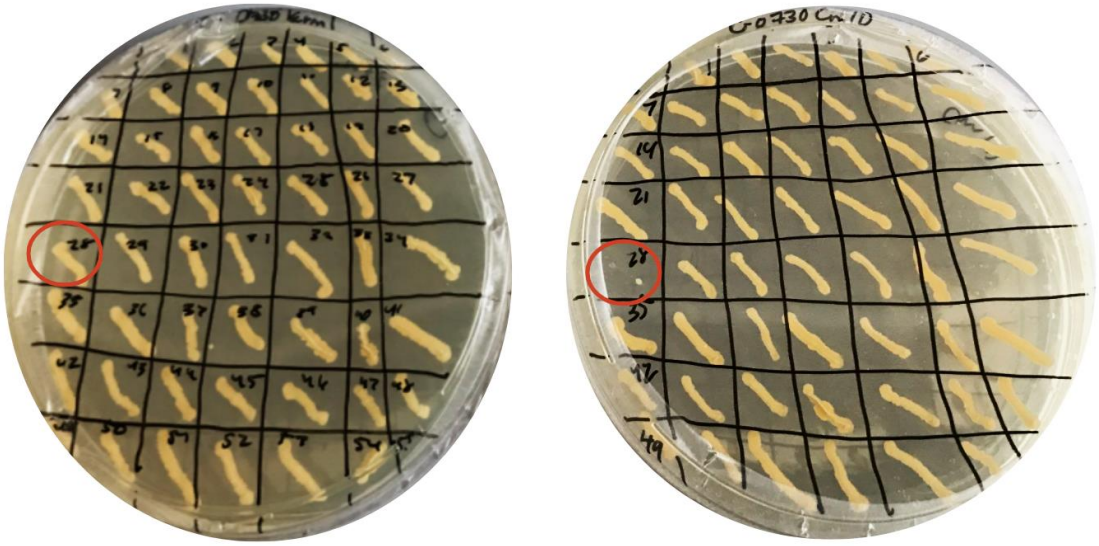


Figure 5. Patched colonies (1-55) on TSA + Erm 5 (left) and TSA + Cm10 (right) for chloramphenicol-sensitive and erythromycin-resistant selection. Circled colony (28) indicates homologous recombination event of 0730 that lost the plasmid but was replaced with erythromycin resistant gene indicating a transposon mutant in *0730*.

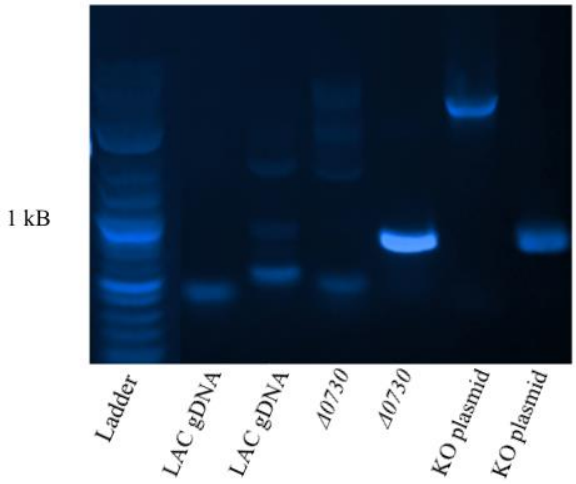
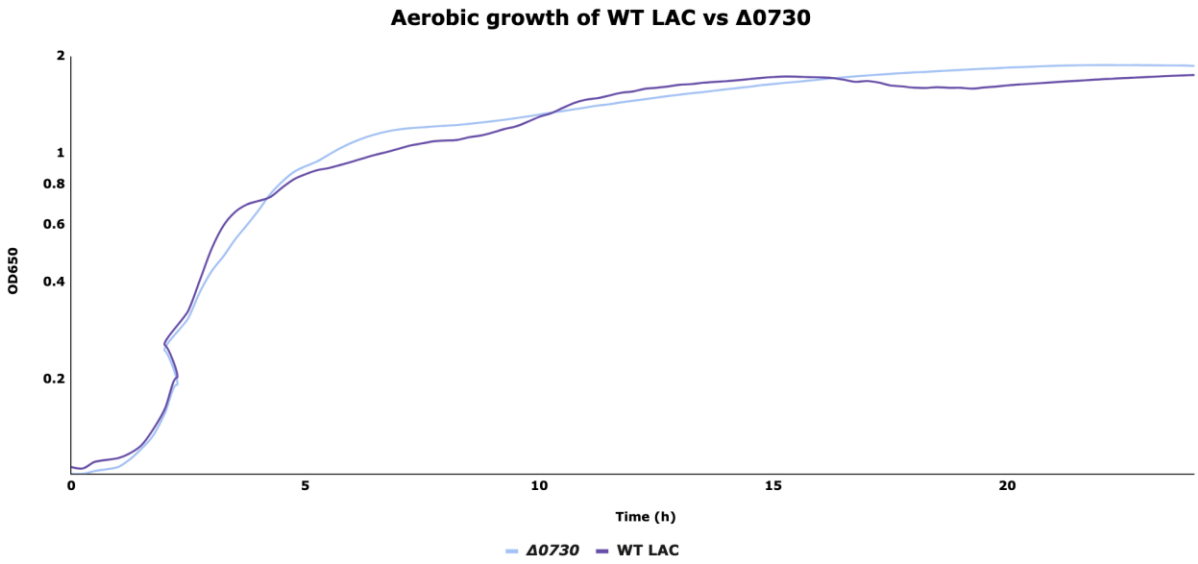


Figure 6. Gel electrophoresis of mutant confirmation of 0730. The first lane for each sample is a PCR done with plasmid-specific primers (as expected WT LAC and the Δ0730 mutant no longer contain the plasmid; all bands in those lanes are nonspecific). The second lane for each sample is a PCR to confirm presence of the *ermB* cassette between the 0730 flanking regions. The mutant shows a product with this PCR despite loss of the plasmid, indicating successful mutant creation and presence of *ermB* in the chromosome.

3.2 *Δ0730* mutant exhibited a slight decrease in fitness relative to WT under NO·

A growth curve was done to compare the growth of the LAC *0730* mutant relative to wild-type LAC under two different concentrations of NO·. The slow release NO· donor DETANO was used for all growth experiments. In our assay, we found that *Δ0730* had a slight defect in growth when exposed to 5 mM NO· as measured by a slightly longer lag time, or time to reach exponential growth while under NO· exposure (Figure 7). Our results suggest that under NO· stress, *Δ0730* had a slight decrease in fitness relative to WT.



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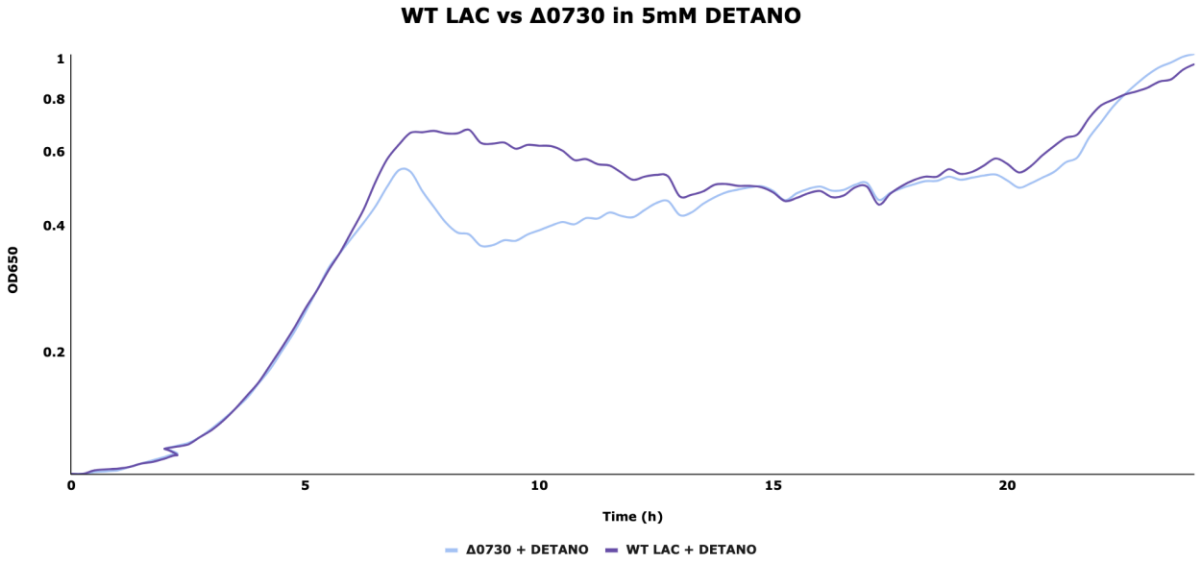


B

Figure 7. *Δ0730* had a slight decrease in growth fitness relative to WT. (n=3 biological replicates grown on 3 different 96 well plates for 24 hours each, done in 3 days). Representative graphs were shown for each growth curve condition.

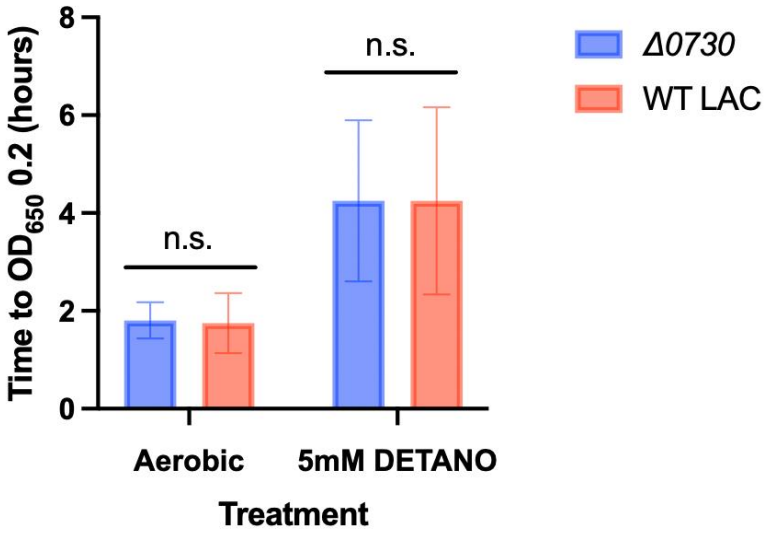
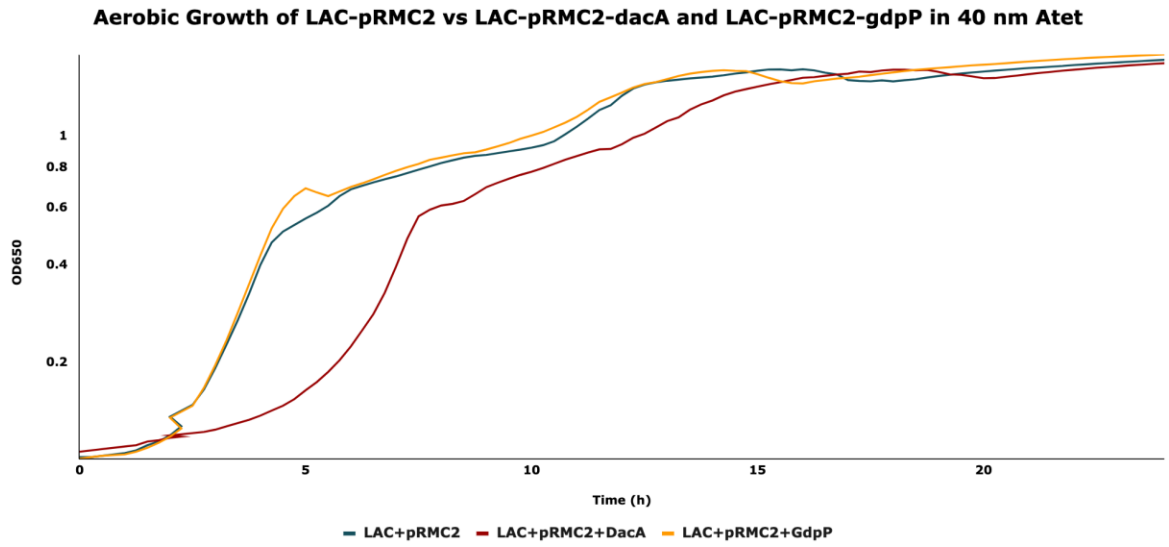


Figure 8. The average time to reach exponential growth (OD650 0.2) of WT LAC and *Δ0730* strains under aerobic growth (Without NO·) or in 5mM DETANO (With NO·). (n=3 biological replicates, performed separately on different days,with 3 replicates of each treatment per day; bar represents mean +/- SEM). A two-way analysis of variance test (ANOVA) with a *post hoc* Bonferroni multiple comparison test revealed no significant (n.s.) difference in the time it took to reach exponential growth of WT LAC vs *Δ0730* in both treatments.

3.3 Over-expression of the enzyme *dacA*, resulting in elevated c-di-AMP levels, is toxic during NO stress.

In addition to examining growth of the deletion mutants, we also performed NO· growth curves on LAC strains with disrupted c-di-AMP levels. These strains contained the tet-inducible plasmid pRMC2 with either *dacA* (encoding c-di-AMP synthesis enzyme) or *gdpP* (encoding c-di-AMP degradation enzyme) downstream of the tet operator. LAC containing empty pRMC2 was used as a vector control strain. The strains containing overexpression plasmids were each tested for NO· sensitivity with DETANO added (as NO· donor) and anhydrotetracycline (atet) used to induce expression from pRMC2. During aerobic growth (no NO·), induction of *dacA* by the addition of 40 nm atet caused a short increase in lag. However, we found that when NO· and atet were added together, cells containing the pRMC2-dacA plasmid never recovered or resumed exponential growth (Figure 9B). This demonstrates that over-production of c-di-AMP almost entirely eliminates the ability of *S. aureus* to grow during NO· stress, suggesting that its proper regulation is critical for its survival. Correspondingly, we found that during NO· stress, the strain LAC pRMC2-gdpP with atet added to induce *gdpP* expression had no significant difference in recovery than the LAC-pRMC2 vector control under the same conditions (Figure 10). Induction of *gdpP* under aerobic conditions did not increase the lag time (Figure 9A). Collectively, these results suggest that lower c-di-AMP levels are beneficial specifically during nitrosative stress.



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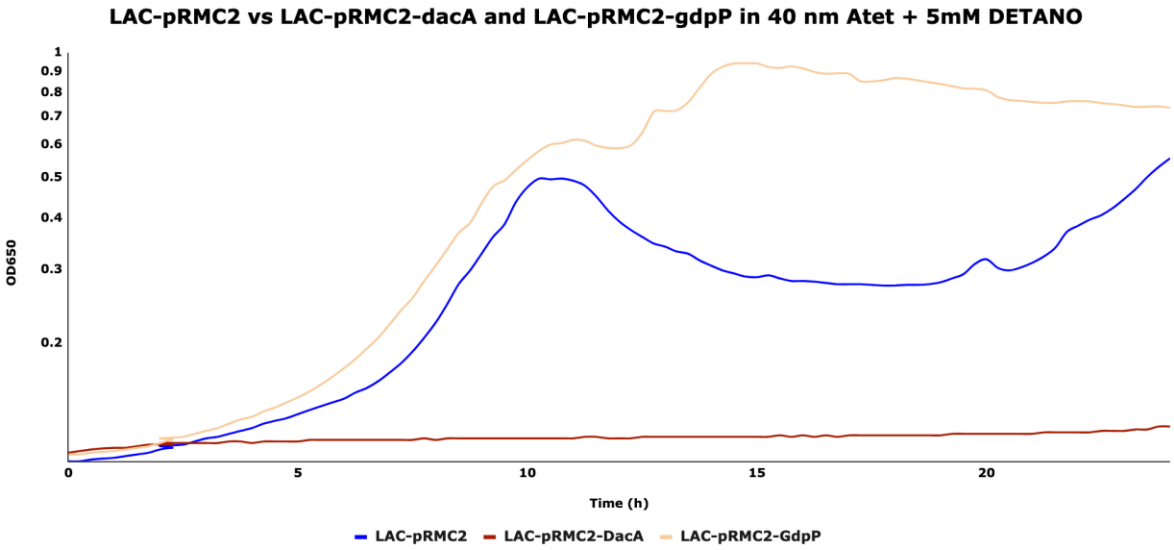

B

Figure 9. Aerobic growth of LAC-pRMC2, LAC-pRMC2+dacA and LAC-pRMC2+gdpP with anhydrotetracycline (atet) added. Induction of *dacA* had a short increase in lag time (A) compared to *gdpP* which had no increase in the lag time (A). Induction of dacA under nitric oxide and atet caused the cells to never fully recover or resume exponential growth (B). LAC-pRMC2+gdpP under nitric oxide and atet addition had an increased recovery rate relative to the vector control (LAC+pRMC2) (B). (n=3 biological replicates grown on 3 different 96 well plates for 24 hours each, done in 3 days; Representative growth curves are shown above).

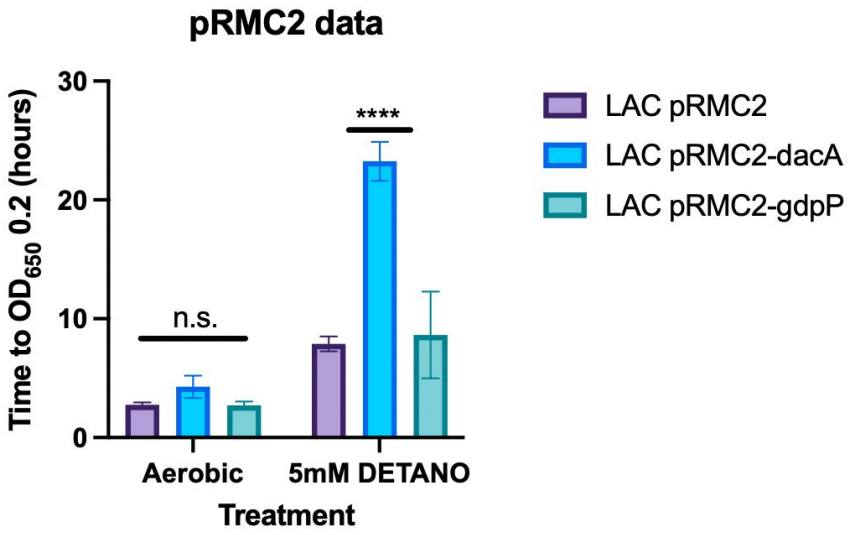

Figure 10. The average time to reach exponential growth (OD650 0.2) of vector control LAC-pRMC2 and overexpression strains (LAC-pRMC2-dacA and LAC-pRMC2-gdpP) under aerobic growth (Without NO·) or in 5mM DETANO (With NO·). (n=3 biological replicates, performed separately on different days,with 3 replicates of each treatment per day; bar represents mean +/- SEM). A two-way analysis of variance test (ANOVA) with a *post hoc* Bonferroni multiple comparison test revealed significance for the LAC-pRMC2-dacA strain in the experiment (***, $p \leq 0.0001$; n.s., not significant).

4. Discussion

Staphylococcus aureus (*S. aureus*) is a successful pathogen containing several crucial virulence factors that make it uniquely able to adapt and proliferate under different stresses imposed by the host immune system such as the radical NO· relative to other bacterial species (Grosser et al., 2016). One of the key components that *S. aureus* uses to continue its pathogenesis is through the help of the second-messenger c-di-AMP. Here, we investigated the role of c-di-AMP levels during nitrosative stress by overexpressing the genes *dacA* and *gdpP* (encoding c-di-AMP synthesis and degradation enzymes, respectively) under an inducible promoter and found that high c-di-AMP levels are toxic during NO· stress. This suggests that proper regulation of high c-di-AMP levels, more specifically keeping levels low, are necessary for *S. aureus* to survive under nitrosative stress. This data is consistent with Corrigan et al, 2011 that proper regulation of normal c-di-AMP levels are critical under stress conditions. However, while one study found that low c-di-AMP levels increase reactive oxygen species (ROS), our study suggests low c-di-AMP levels are beneficial during nitrosative stress (Corrigan et. al., 2011; Zeden et al., 2018). This could be explained by the fact that nitrosative and oxidative stress impact bacteria in very different (though sometimes overlapping) ways. In addition, a SAUSA300_0730 transposon mutant was investigated and characterized under NO· stress conditions and our results suggest that the *0730* mutant exhibited a phenotype of a slight decrease in fitness relative to wild-type. This suggests that a clean deletion in *Δ0730*, causes *S. aureus* to perform worse under nitric oxide stress indicating that it is a crucial gene involved in nitric oxide resistance.

In addition to generating a 0730 transposon mutant, attempts were made to generate a deletion mutant in PstA. The deletion of *pstA* was constructed and is undergoing further testing to confirm that it is indeed a mutant. Once the deletion mutant *pstA* is confirmed via PCR, it will undergo growth curves to characterize its phenotype during nitrosative stress.

Future work will be done on detecting the c-di-AMP levels in WT *S. aureus* LAC and *Δ0730* during NO· stress using an already constructed Spinach2-based Biosensor, to investigate how levels normally fluctuate during NO· stress. In addition, to evaluate the changes in gene expression during NO· stress that are impacted by disrupted c-di-AMP levels, GFP reporter plasmids will be inserted into the *dacA* and *gdpP* overexpression strains for the known NO· resistance genes *Hmp* and *Ldh1*. The gene *hmp is a* conserved flavohemoprotein that detoxifies NO· by turning it into

nitrate, but it is not known what upregulates this gene to quickly help *S. aureus* detoxify NO· (Richardson et al., 2006; Richardson et al., 2008). Also during NO· stress, *S. aureus* uses a gene *Ldh1*, lactate dehydrogenase to allow fermentation and continue growing. By inserting a GFP reporter for *hmp* and *ldh1* expression, this will allow us to investigate in real-time whether disrupting c-di-AMP levels affects the expression of *hmp* or *ldh1*. Furthermore, it would be noteworthy to determine how global gene expression is impacted during altered c-di-AMP levels during NO· stress by performing RNA-Seq. To do so, a large culture of the *S. aureus* WT vector control and each of the dacA and gdpP overexpression strains will be exposed to NO· and RNA will then be purified and collected from each sample and sent out for RNA sequencing. Altogether, our findings provide some insights into the importance of c-di-AMP signaling under nitrosative stress and this could help identify potential antibiotics to disrupt this signaling pathway in *S. aureus* to provide new therapeutic treatments.

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