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Creating and Evaluating Two Potential cyclic-di-AMP Biosensors in *Staphylococcus aureus*

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Abstract

Staphylococcus aureus is a ubiquitous Gram-positive human pathogen with antibiotic resistant characteristics. It is the culprit for a majority of skin and soft tissue infections (SSTIs), which can lead to more serious infections such as endocarditis. The success of *S. aureus* can be traced to several key virulence traits, including resistance to nitric oxide (NO·), a crucial component of the human innate immune response. *S. aureus* promotes a metabolic state in the presence of NO· to continue replication and detoxify NO·. Furthermore, *S. aureus* utilizes a second messenger signaling system that relies on the small molecule cyclic di-adenosine monophosphate (c-di-AMP), which regulates cell wall size and other basic cellular processes such as respiration, there is evidence that it may also play a role in NO· resistance. Developing methods to detect concentrations of c-di-AMP during cell stress will lead to further understanding of the role this second messenger plays during the pathogenesis of *S. aureus*. On a cellular level, c-di-AMP binds to protein and RNA effectors that alter transcription, translation, or enzyme function. Linking c-di-AMP-responsive protein or RNA effectors to conditionally-fluorescent molecules has been demonstrated as a viable strategy for c-di-AMP detection in another pathogen, *Listeria monocytogenes.* We constructed and tested several genetically encoded biosensor plasmid constructs in *S. aureus* using a microplate reader. Our data evaluate the efficacy of each biosensor for detecting changes in c-di-AMP levels that are induced by genetic manipulation. We show that differences can be detected, but further optimization is needed to improve sensitivity. Understanding the conditions under which c-di-AMP levels fluctuate in *S. aureus* ultimately creates a stronger understanding of its function in bacteria and role in NO· resistance in *Staphylococcus aureus*. Additionally, the successful creation of c-di-AMP biosensors in *S. aureus* will allow future screens of chemical compounds that alter c-di-AMP levels in bacteria as potential new drugs.

1. Introduction

Staphylococcus aureus can be found either permanently or intermittently in the nares and on the skin of humans. Its infections can range in severity from skin infections to bacteremia and are becoming increasingly difficult to treat due to the evolution of drug-resistant strains[1]. The persistence of *S. aureus* is partially attributed to its resistance to nitric oxide (NO·), an antimicrobial produced by the innate immune system that is important for reducing replication of bacteria[2]. The consequences of NO· exposure to a bacterial cell are immense: DNA damage; growth inhibition; cell death; and destruction of metal centers of bacterial enzymes such as heme iron, iron-sulfur clusters, and other transition metal cofactors[2]. When NO· reacts with oxygen, it creates a myriad of toxic reactive oxygen species (ROS) that have the ability to target and damage bacterial DNA, which leads to cell death unless the toxic species are quickly detoxified and any damage is rapidly repaired[3]. The ability of *S. aureus* to resist NO· and continue to proliferate in its presence is an impressive and extraordinary trait in the bacterial world. The results of a Tn-Seq screen, which determines quantitative fitness of a pool of transposon mutants, identified three genes as being required for NO· resistance that relate to cyclic di-adenosine monophosphate (c-di-AMP) signaling (*ybbR, pstA*, and SAUSA300_0730), this suggests

that cyclic di-adenosine monophosphate (c-di-AMP) may be involved in the *S. aureus* response to NO·[2]. The gene *ybbR* shares an operon with *dacA* and physically interacts with it in the membrane, however, its true qualities remain unknown[2]. Secondly, *pstA* is a known protein target of c-di-AMP, but little else is known thus-far[2]. Lastly, SAUSA300_0730, yet to be officially named, shares a domain with *dacA*, which suggests that it may either synthesize, degrade, or interact with a di-nucleotide such as c-di-AMP[2].

c-di-AMP is a second messenger molecule. Second messengers are used by bacteria to quickly react and adapt to changes in their environment and have transcriptional, post-transcriptional, and post-translational regulatory mechanisms[4]. c-di-AMP is synthesized by the enzyme DacA and is degraded by the enzyme GdpP; both enzymes are crucial for the viability and growth of *S. aureus*[4]. Cyclic di-AMP is involved in regulation of cell size and cell wall stress tolerance, and there is evidence that it stimulates the response of the innate immune system in mammalian cells[5]. In *S. aureus*, decreased levels of cyclic-di-AMP result in increased ROS toxicity. Because ROS damage overlaps with the effects of NO· stress, it may be especially important to appropriately modulate c-di-AMP levels during NO· stress[4]. Additionally, decreased levels of c-di-AMP reduce cell size and resistance to bacterial cell wall targeting antibiotics, which makes the second messenger pathway worthy of note from a drug-targeting perspective[5]. Along with other second messenger systems, c-di-AMP signaling appears to be strictly controlled both temporally and spatially, underscoring the crucial need for *in vivo* biosensors that can monitor how and when physiological changes occur[5]. Current c-di-AMP detection methods include enzyme immunoassays, several of which are limited to *in vitro* detection, such as mass spectrometry, but to further investigate the role of c-di-AMP in pathogenesis a vigorous method for real-time, kinetic, and *in vivo* detection of cyclic-di-AMP is required[5].

In *Listeria monocytogenes*, another Gram-positive pathogen, *yuaA* is a c-di-AMP responsive riboswitch, which is a segment of an mRNA molecule that binds to small molecules and changes structure (and subsequently transcription or translation efficacy) in response to binding[6]. A c-di-AMP biosensor was constructed for *L. monocytogenes* by fusing the RNA aptamer Spinach2 with *yuaA*. Spinach2 is a genetically encoded RNA molecule that fluoresces when bound to the small molecule DFHBI. The yuaA-Spinach2 fusion was constructed so that Spinach2 can only fold into its fluorescent conformation when c-di-AMP is also bound to *yuaA*. The brightness of fluorescence is dependent upon the concentration of c-di-AMP[7]. In this 2015 study, a plasmid encoding the *yuaA*-Spinach2 biosensor was transformed into *Listeria* to detect c-di-AMP in live cells, and the biosensor produced a fluorescent readout for c-di-AMP concentration that differed in mutant versus wild-type strains[7]. In addition to the *yuaA* biosensor, Woodward and colleagues recently created a second *L. monocytogenes* biosensor that exhibits fluorescence due to Förster resonance energy transfer (FRET), called CDA5. This new biosensor is also non-destructive and specific, and it can detect c-di-AMP dynamics both *in vitro* and *in vivo*. For this sensor, a truncated version of the *L. monocytogenes* c-di-AMP-binding protein Lmo0553 was fused to both CFP and YFP fluorescent proteins. When Lmo0553 binds c-di-AMP, it changes shape so that the CFP and YFP arms are brought close together. The CFP is then excited with a specific wavelength of light (425nm) and will emit light at another wavelength; if in close proximity to YFP, it will transfer energy to the YFP and the overall emission (535nm) spectrum will be different than if the two are far apart from each other.

To modify the Spinach2 biosensor for expression in *S. aureus*, two different *S. aureus* constitutive promoters, *rpoD* and *rpsJ*, were chosen for expression of the *yuaA*-Spinach2 fusion since it has only ever previously been expressed in *L. monocytogenes* with *Listeria* promoters. Two different *S. aureus* plasmid backbones, pRMC2 and pCN52, with slightly different copy numbers were also investigated. As a control, Spinach2 was fused to a tRNA platform, instead of *yuaA*, which does not bind with c-di-AMP and therefore should not exhibit a change in fluorescence with fluctuating c-di-AMP levels. Separately, two plasmids were created that allow inducible expression of *gdpP* and *dacA* to manipulate levels of c-di-AMP in *S. aureus*. These were combined into strains that also contained the *yuaA*-Spinach2 biosensor construct so that biosensor activity could be monitored as c-di-AMP levels were manipulated. These were all used in tandem to determine which of the possibilities among the plasmids allowed for the most sensitive detection of c-di-AMP levels. The CDA5 biosensor may have several advantages over the *yuaA*-Spinach2 biosensor. Spinach2 relies on fluorescent ligand (DFHBI) uptake by bacterial cells for fluorescence, which makes it susceptible to potentially variable uptake, and it lacks an internal control[8]. The CDA5 biosensor was also expressed in *Bacillus subtilis* in Choi et al., 2021 and successfully detected c-di-AMP levels in live *B. subtilis* cells, so it is likely to work in *S. aureus* as well.

The importance of c-di-AMP in *S. aureus* is evident, and the hypothesis is that a biosensor can be developed to determine if, when, how long, and how much cyclic-di-AMP is activated under certain stressors in *S. aureus*, especially during NO· stress. Furthermore, a fully developed and reliable biosensor can be used to screen chemical compounds to identify drugs that target *dacA* or *gdpP* to alter c-di-AMP levels in *S. aureus*. Altogether, this information could help to learn more about c-di-AMP signaling pathways in *S. aureus*, and potentially to identify new drugs that interfere with c-di-AMP signaling.

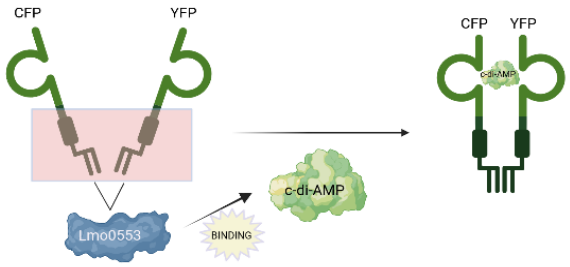

Figure 1. Förster resonance energy transfer (FRET) configuration of CDA5.

Figure 1. The c-di-AMP-binding protein, Lmo0553, was fused to both CFP and YFP fluorescent proteins. When Lmo0553 binds to c-di-AMP, it changes shape so that the CFP and YFP arms 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 (535nm).

2. Materials and Methods

2.1 Construction of Biosensor Vectors

For the construction of 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. The Spinach-*yuaA* and Spinach-tRNA biosensors were synthesized by Twist Bioscience and cloned into the KpnI and PstI restriction sites of each vector downstream of the constitutive promoters. The Spinach2 biosensor was then transformed into NEB DH5**α** competent *E.coli* cells using pRMC2 and pCN52 plasmids. Next, the cultures were plated on Lysogeny broth (LB) agar + 100µg/ml of the antibiotic ampicillin (Amp 100), and placed in an incubator at 37℃ overnight. The resulting strains were: DH5**α**-pCN52-*rpOD-yuaA*, DH5**α**-pCN52-*rpOD*-tRNA, DH5**α**-pCN52-*rpSJ-yuaA*, DH5**α**-pCN52-*rpSJ*-tRNA, DH5**α**-pRMC2-*rpOD-yuaA*, DH5**α**-pRMC2-*rpOD*-tRNA, DH5**α**-pRMC2-*rpSJ-yuaA*, and DH5**α**-pRMC2-*rpSJ*-tRNA.

Plates were inspected and two colonies from each plate were chosen at random. A colony PCR was performed using the standard OneTaq protocol (initial denaturation at 94℃ for 30 seconds, 30 cycles at 94℃ for 15-30 seconds, 45-68 ℃ for 15-60 seconds, 68℃ for 1 minute per kilobases, final extension at 65℃ for 2 minutes, and infinite hold at 4-10℃). This was performed to confirm that the inserts, *yuaA*-Spinach2 or tRNA-Spinach2, were present and in correct orientation. Insert-specific primers were purchased from Eurofins Genomics for *yuaA*-Spinach2 or tRNA-Spinach2 to confirm the inserts. Colonies that indicated a ~337bp PCR product for *yuaA*-Spinach2 and ~238bp for tRNA-Spinach2 via gel electrophoresis, were grown in test tubes containing 5.0 mL LB liquid media with 5.0µL Amp 100 and grown in an incubator at 37℃ overnight. Freezer stocks were made from 500.0µL overnight liquid cultures and 500.0µL of 50% glycerol, labeled, then stored in a -80℃ freezer. The plasmids were extracted using a IBI High-Speed Plasmid Mini Kit to later be used to transform *S. aureus* 'RN4220'.

The CDA5 biosensor was synthesized from Twist Bioscience into the Twist high-copy vector plasmid. The biosensor sequence was modified from the *Listeria monocytogenes* version in Choi et al. Next, the biosensor fragment was amplified from the Twist vector by PCR, which was performed in a T100 thermal cycler using NEB Q5 High-Fidelity DNA Polymerase and a touchdown (TD) protocol (initial denaturation at 98℃ for 30 seconds, 25-35 cycles at 98℃ for 5-10 seconds, 50-72℃ for 20-30 seconds, 72℃ for 20-30 per kilobases, final extension at 72℃ for 60

seconds, and infinite hold at 4-10℃). A gel electrophoresis, conducted using standard protocols, was performed to confirm PCR success.

2.2 Transformation of Biosensor Vectors into *S. aureus*

S. aureus 'RN4220' has a mutation in the *sau1 hsdR* gene, resulting in a restriction deficiency, which facilitates uptake of *E. coli* DNA; it methylates it appropriately, 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. Following electroporation, cultures were plated on Tryptic Soy Agar (TSA) plates with 5µg/ml of the antibiotic erythromycin (Erm 5) and 10µg/ml of the antibiotic chloramphenicol (Cm 10), then placed in an incubator overnight at 37°C. Successful colonies were chosen and grown in a test tube that contained 5.0 mL of liquid media Tryptic Soy Broth (TSB) and 5.0µL of Erm 5 and Cm 10, and placed in an incubator overnight at 37°C. Freezer stocks were made from 500.0µL overnight liquid cultures and 500.0µL of 50% glycerol, labeled, then stored in a -80℃ freezer.

Next, the overnight cultures were miniprepped using the standard NEB IBI miniprep protocol, with an additional step of lysostaphin treatment (2.5mg/ml) following PD1 buffer resuspension to lyse the *S. aureus* RN4220 cell wall. The miniprep product was transformed into two strains of community acquired methicillin-resistant *S. aureus* (MRSA), called 'LAC' - LAC-*gdpP* and LAC-*dacA*, which were made electrocompetent using the same standard procedure as described above. These strains contained the plasmids pRMC2-*dacA* and pRMC2-*gdpP*, in which *dacA* or *gdpP* expression is controlled by an anhydrotetracycline (aTc)- inducible promoter, both of which were created previously by a fellow undergraduate researcher, Michelle Angeles. aTc is a derivative of the antibiotic tetracycline that is used for studying tetracycline-controlled gene expressions in bacteria as it does not exhibit antibiotic activity. The transformation resulted in the following strains of LAC: *dacA*-pCN52-*rpOD*- *yuaA*-Spinach2, *gdpP*-pCN52-*rpOD*- *yuaA*-Spinach2, *dacA*-pCN52-*rpSJ*- *yuaA*-Spinach2, and *gdpP*-pCN52-*rpSJ*- *yuaA*-Spinach2, with their respective control tRNA-Spinach2 counterparts.

The cultures were plated on TSA + Erm 5 and Cm 10, and placed in an incubator overnight at 37°C. Successful colonies were chosen and grown in a test tube that contained 5.0 mL of liquid media Tryptic Soy Broth (TSB) and 5.0µL of Erm 5 and Cm 10, and placed in an incubator overnight at 37°C. Freezer stocks were made from 500.0µL overnight liquid cultures and 500.0µL of 50% glycerol, labeled, then stored in a -80℃ freezer.

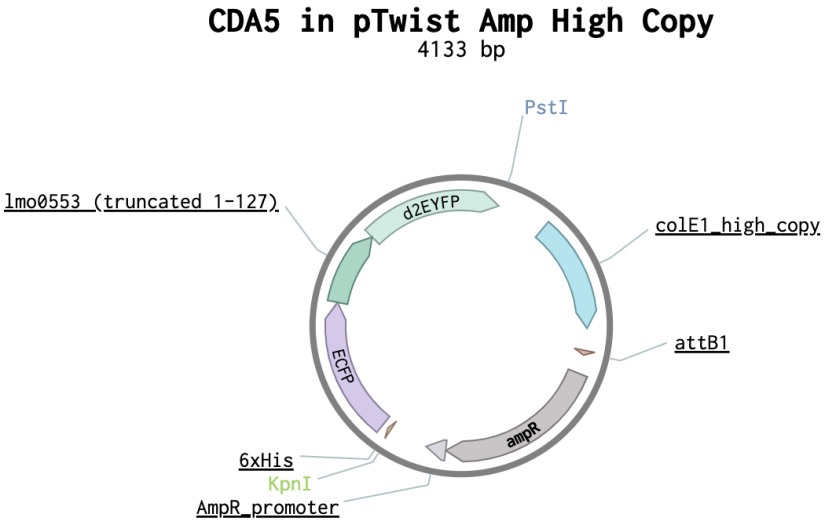

Figure 2. The CDA5 Twist Bioscience Biosensor.

Figure 2. The c-di-AMP-binding protein, Lmo0553, ("truncated 1-127") with the fluorescent proteins CFP and YFP flanking its sides. Restriction sites for KpnI and PstI are also indicated lying outside of the fluorescent proteins.

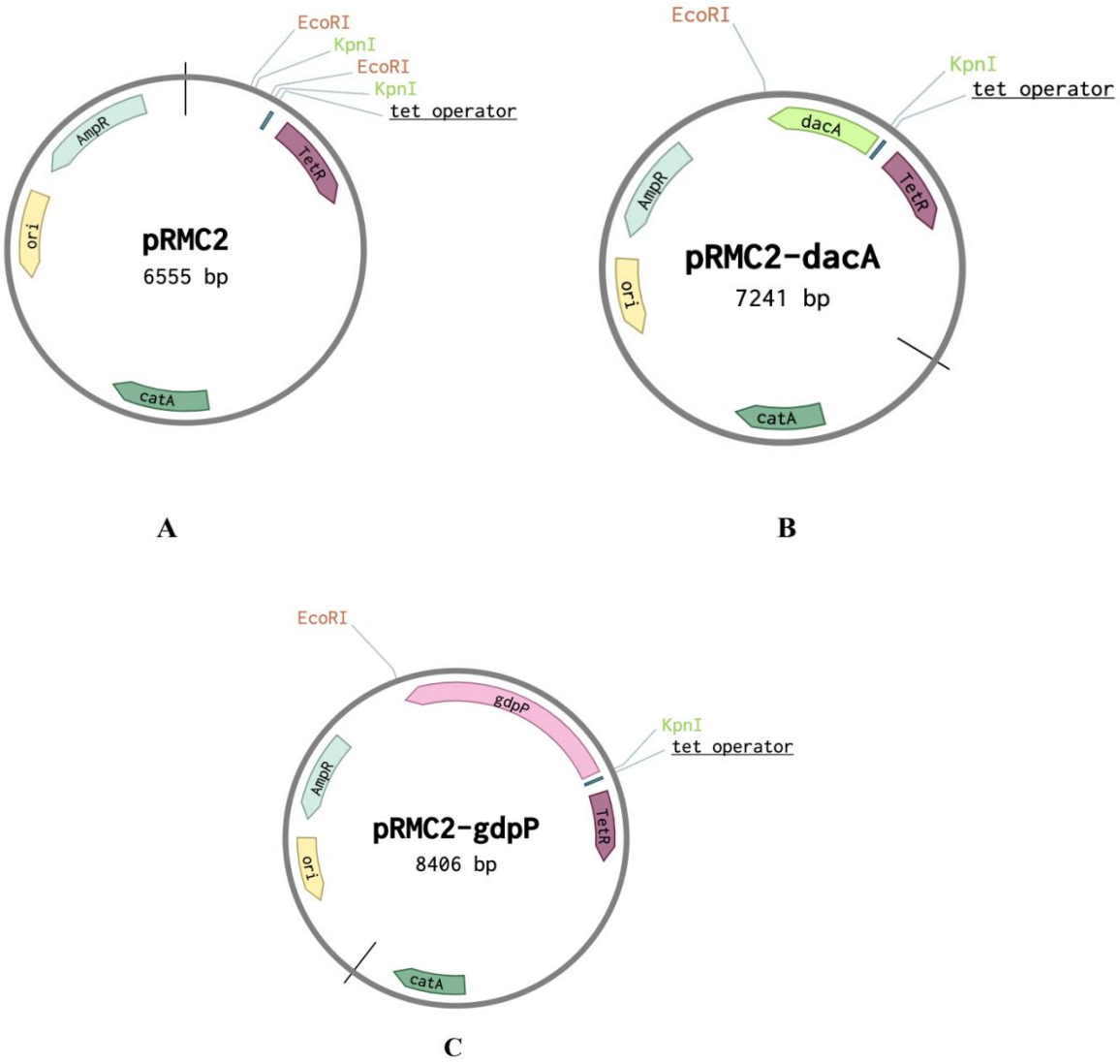

Figure 3. pRMC2 backbone plasmids.

Figure 3. Vector control (**A**), plasmid containing an overexpression of *dacA* to synthesize c-di-AMP (**B**) and *gdpP* to degrade c-di-AMP (**C**).

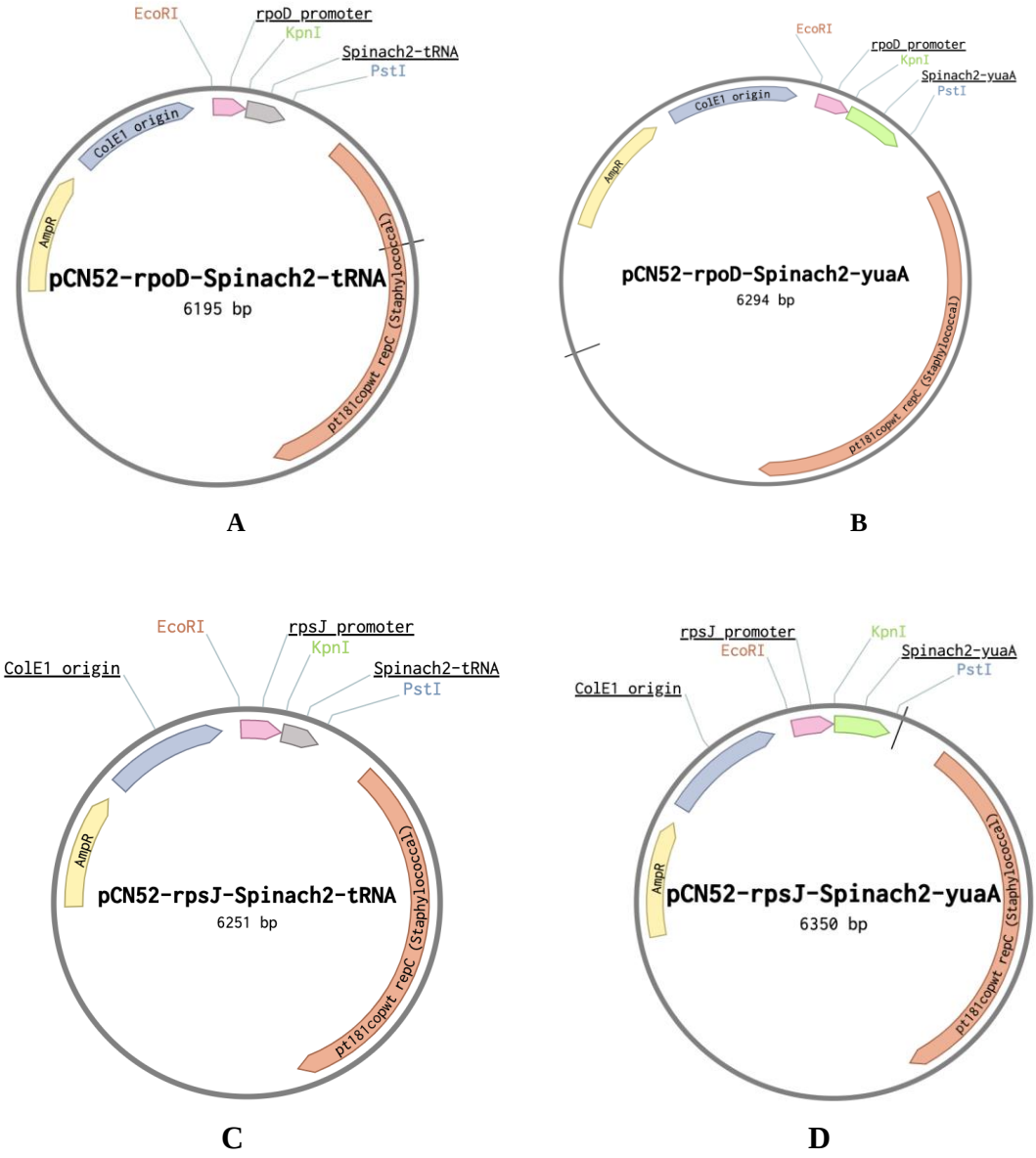

Figure 4. pCN52 backbone plasmids.

Figure 4. Plasmid containing the *rpOD* promoter and tRNA-Spinach2 as a control (**A**), plasmid with the *rpOD* promoter and *yuaA*-Spinach2 biosensor (**B**), plasmid containing the *rpSJ* promoter and tRNA-Spinach2 control (**C**), and the plasmid with the *rpSJ* promoter and *yuaA*-Spinach2 biosensor (**D**).

2.3 Growth Curve Assays

To test if a controlled increase or decrease in c-di-AMP could be detected with the *yuaA*-Spinach2 biosensor, the induction of *dacA* expression from the plasmid pRMC2-*dacA* was used to increase c-di-AMP levels, and the induction of *gdpP* from the plasmid pRMC2-*gdpP* was used to decrease c-di-AMP levels. From these plasmids, *dacA* and *gdpP* expression was induced by the addition of a range of aTc.

Strains chosen for experimentation were taken out of a -80°C storage freezer and inoculated into 5.0 mL TSB along with the appropriate antibiotic(s), and placed in an incubator overnight at 37°C. The following day, cultures were diluted to a final OD_{650} reading of 0.01. Depending upon which biosensor was being tested, 2.0µL of antibiotic(s) was added to the final dilution set for each strain, e.g. Spinach2 received 2.0µL of 10µg/ml Cm 10 and 5µg/ml Erm

5. In the 96 well plate, 200.0µL of each dilution was placed into their respective wells according to the experiment setup designed previously. Fluorescence from the biosensors was detected using a Biotek Synergy H1 microplate reader to detect fluorescence of batch cultures in 96-well plates, 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: 830 seconds shaking, orbital mode, 1mm amplitude; 2nd shaking: 30 sec duration, linear mode, 1mm amplitude, 10 sec settle time, 5 flashes). The average fluorescence for the whole culture, with the excitation and emission for Spinach2 set to 485nm and 528nm[12].

The OD_{650} reading of each culture was carefully monitored, when the average OD_{650} reached 0.15 the process was halted in order to add various amounts of aTc and DFHBI to each well in accordance with the experimental design created previously. A 2µg/ml solution of aTc was created in dimethylsulfoxide (DMSO), which was then added at a final concentration of 20-60ng/ml to *S. aureus* cultures. A 20mM working stock solution of (Z)-4-(3,5-difluoro-4-hydroxybenzylidene)-1,2-dimethyl-1H-imidazol-5(4H)-one (DFHBI) was created in DMSO and added at a final concentration of 200µm to *S. aureus* cultures. Both aTc and DFHBI were placed in 2.0mL microcentrifuge tubes, wrapped in aluminum foil due to their photosensitivity, and placed in storage boxes. aTc was stored in a freezer at 0°C, and DFHBI was stored in a refrigerator at 5°C.

The sensitivity of the biosensors were tested by using the range of aTc to induce a spectrum of c-di-AMP levels to determine dose-dependent responses of the biosensors. Once completed, the 96 well plate was placed back into the BioTek reader and the process continued until the 24 hour mark was reached and the experiment thus ended. Relative fluorescence units were normalized to the approximate culture density by dividing by OD_{650} at each timepoint. A two-way ANOVA was used to compare normalized fluorescence between the control tRNA-Spinach2 plasmid and pRMC2-*dacA* plasmid.

3. Results

3.1 *yuaA* Biosensor Displayed Higher Average Fluorescence Values Relative to tRNA Control

Growth assays in TSB were conducted to compare the overall fluorescence of LAC *dacA*-pCN52-*rpOD-yuaA*, *dacA*-pCN52-*rpOD*-tRNA, *dacA*-pCN52-*rpSJ-yuaA*, and *dacA*-pCN52-r*pSJ*-tRNA cultures. Cultures were exposed to varying quantities of aTc to induce *dacA* expression and increase c-di-AMP levels over a 24 hour period. Cultures containing the *yuaA*-Spinach2 biosensor produced significantly more fluorescence than the tRNA-Spinach2 control when c-di-AMP levels were elevated via *dacA* induction, indicating successful biosensor activity (Figure 5a and 5b).

dacA-pCN52-*rpOD-yuaA* and *dacA-pCN52-rpOD*-tRNA, there was a significant difference in fluorescence between these two strains (two-way ANOVA; Interaction: $F(2, 30) = 2.144$, $p=0.1348$; Row Factor (aTC concentration): $F(2, 30) = 1.3$, $p=0.2874$; Column Factor (strain): $F(1, 30) = 8.848$, $p=0.0057$) at both the 20ng/ml and 40ng/ml concentrations of aTc (post-hoc Bonferroni multiple comparisons test).

dacA-pCN52-*rpSJ-yuaA* and *dacA-pCN52-rpSJ*-tRNA, there was a significant difference in fluorescence between these two strains (two-way ANOVA; Interaction: $F(2, 30) = 3.292$, $p=0.0510$; Row Factor (aTC concentration): $F(2, 30) = 6.47$, $p=0.0046$; Column Factor (strain): $F(1, 30) = 9.866$, $p=0.0038$) at both the 20ng/ml and 40ng/ml concentrations of aTc (post-hoc Bonferroni multiple comparisons test).

3.2 Differences of Fluorescent Values between *rpOD-yuaA* and *rpSJ-yuaA* Cultures

Next, we compared fluorescence between yuaA-Spinach2 biosensors driven by the *rpoD* versus *rpsJ* promoters. Further growth assays using TSB media were conducted to compare the fluorescence of *dacA*-pCN52-r*pOD-yuaA* and *dacA*-pCN52-*rpSJ-yuaA* LAC cultures. Cultures were exposed to varying quantities of aTc to induce dacA expression and increase c-di-AMP levels over a 24 hour period. From these assays, it was discovered that yuaA-Spinach2 cultures grown in TSB that contained the *rpOD* promoter produced a comparable amount of fluorescence to the cultures containing the *rpSJ* promoter (Figure 5c), suggesting similar levels of biosensor expression from either promoter. Two-way ANOVA; n.s.

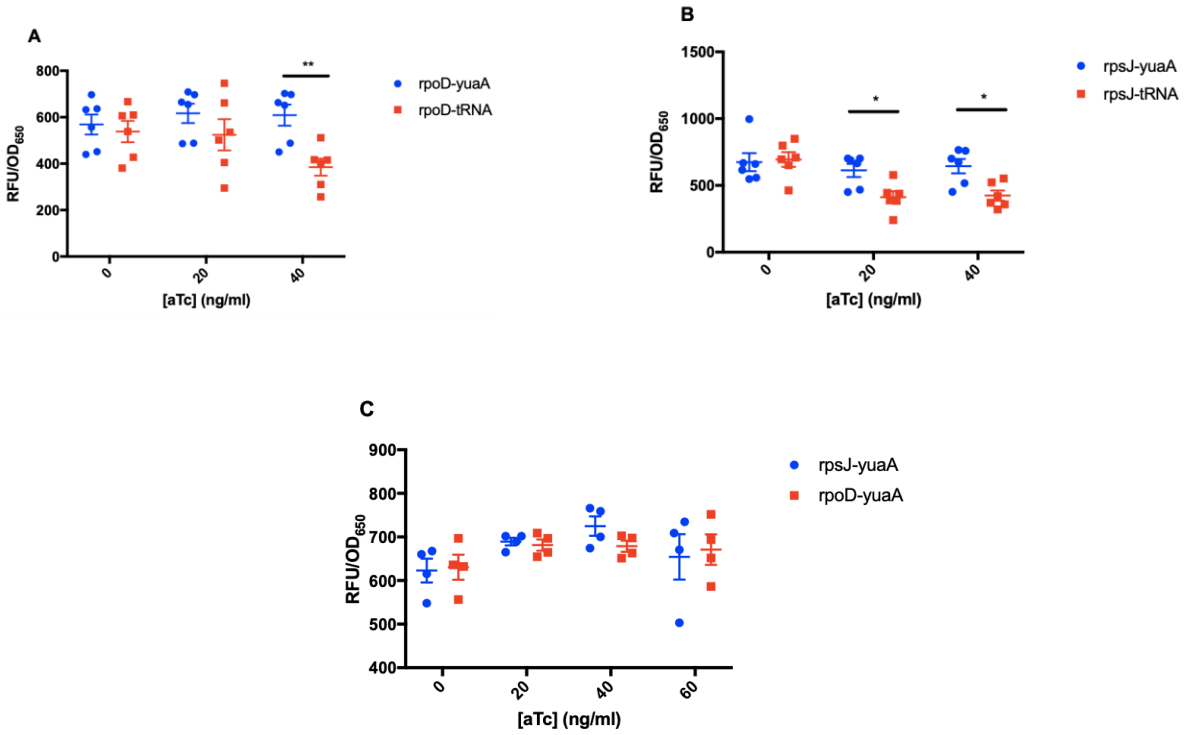

Figure 5. Results of aerobic growth assays.

Figure 5. Final fluorescence values expressed as Relative Fluorescence Units (RFUs) over OD_{650}. (**A**) *dacA*-pCN52-*rpOD-yuaA* (blue) and *dacA-pCN52-rpOD*-tRNA (red) grown in 2.0µL of 20mM DFHBI and varying amounts of aTc ranging 20-60ng/ml at 10.0 hours of growth. There was a significant difference in fluorescence between these two strains (two-way ANOVA; Interaction: F(2, 30) = 2.144, p=0.1348; Row Factor (aTC concentration): F(2, 30) = 1.3, p=0.2874; Column Factor (strain): F(1, 30) = 8.848, p=0.0057) at both the 20 and 40ng/ml concentrations of aTc (post-hoc Bonferroni multiple comparisons test; ** = p<0.01). n=6 biological replicates, performed separately on different days, with 2 technical replicates each day; bars represent mean +/- SEM. (**B**) *dacA*-pCN52-*rpSJ-yuaA* (blue) and *dacA-pCN52-rpSJ*-tRNA (red) grown in 2.0µL of 20mM DFHBI and varying amounts of aTc ranging 20-60ng/ml at 10.0 hours of growth. There was a significant difference in fluorescence between these two strains (two-way ANOVA; Interaction: F(2, 30) = 3.292, p=0.0510; Row Factor (aTC concentration): F(2, 30) = 6.47, p=0.0046; Column Factor (strain): F(1, 30) = 9.866, p=0.0038) at both the 20 and 40ng/ml concentrations of aTc (post-hoc Bonferroni multiple comparisons test; * = p<0.05). n=6 biological replicates, performed separately on different days, with 2 technical replicates each day; bars represent mean +/- SEM. (**C**) *dacA*-pCN52-*rpSJ-yuaA* (blue) and *dacA-pCN52-rpOD-yuaA* (red) grown in 2.0µL of 20mM DFHBI and varying amounts of aTc ranging 20-60ng/ml at 10.0 hours of growth. (n=4 biological replicates, performed separately on different days, with 2 technical replicates; bar represents mean +/- SEM). A two-way analysis of variance test (ANOVA) performed; n.s.

3.3 Anhydrotetracycline Additive Affects Aerobic Growth of Spinach2 Biosensor

We also investigated whether the plasmids and growth conditions used in this study impacted the normal growth of *S. aureus*. Ideally a successful biosensor would not impact growth; however, plasmid maintenance imposes an extra metabolic burden on bacteria, high c-di-AMP levels could be toxic, and DFHBI and/or aTc could be toxic at high concentrations. OD_{650} values of *dacA*-pCN52-*rpOD-yuaA*, *dacA-pCN52-rpOD*-tRNA, *dacA*-pCN52-*rpSJ-yuaA*, and *dacA-pCN52-rpSJ*-tRNA LAC cultures were evaluated after the growth assays using TSB media were completed. Cultures were grown in standard concentrations (20mM) of DFHBI and aTc, with 2.0µL of DFHBI and varying quantities of aTc over a 24 hour period. From the assays, it was discovered that *yuaA*-Spinach2 cultures with the *rpOD* promoter that were exposed to 40ng/ml of aTc had a slightly higher fitness over time compared to the *rpOD*-tRNA cultures within the same parameters (Figure 6). Interestingly, when similar values were compared to the *yuaA*-

Spinach2 and tRNA-Spinach2 cultures with the *rpSJ* promoter, it was discovered that the *rpSJ-yuaA* cultures exposed to 40ng/ml of aTc had a lower fitness compared to the *rpSJ*-tRNA cultures within the same parameters (Figure 7).

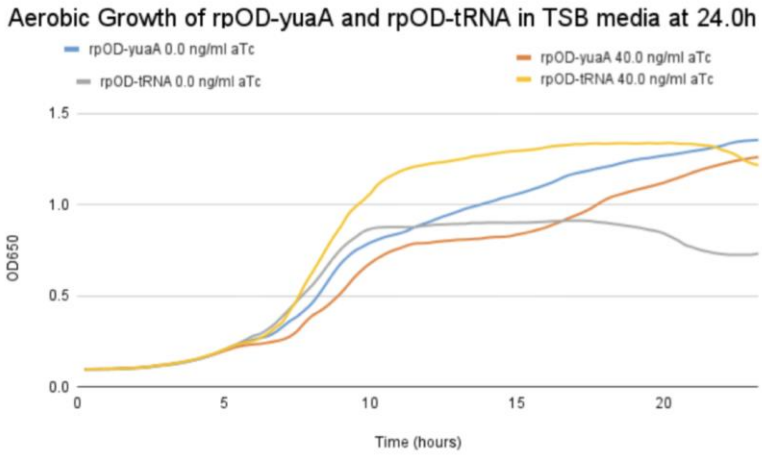

Figure 6. Aerobic growth comparison.

Figure 6. *dacA*-pCN52-*rpOD-yuaA* and *dacA-pCN52-rpOD*-tRNA over a 24 hour period in TSB media and 2.0µL of 20mM DFHBI. The *yuaA*-Spinach2 cultures (orange and blue) show a higher fitness than their tRNA-Spinach2 controls (yellow and gray). This graph is a representative of trends observed in data (n=2 biological replicates, performed separately on different days, with 2 technical replicates).

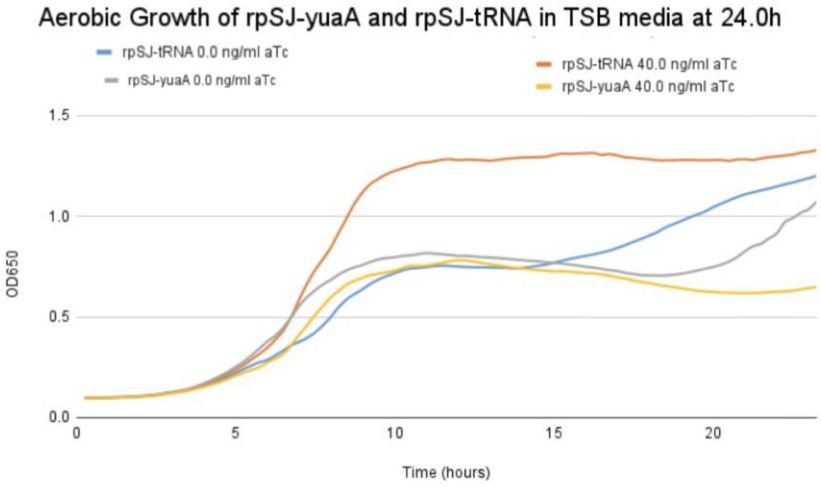

Figure 7. Aerobic growth comparison.

Figure 7. Aerobic growth of *dacA*-pCN52-*rpSJ-yuaA* and *dacA-pCN52-rpSJ*-tRNA over a 24 hour period in TSB media and 2.0µL of 20mM DFHBI. The *yuaA*-Spinach2 cultures (yellow and gray) show a slightly lower fitness than their tRNA-Spinach2 controls (blue and orange). This graph is a representative of trends observed in data (n=2 biological replicates, performed separately on different days, with 2 technical replicates).

4. Discussion

Staphylococcus aureus (*S. aureus*) is able to overcome and propagate under innate host immune responses due to an adaptive arsenal of different virulence mechanisms that are not widely observed in other bacteria. c-di-AMP, a second messenger, is a crucial instrument that aids in the pathogenesis of *S. aureus.* Here, we detected the presence of c-di-AMP levels during aerobic growth under antibiotic stress by using the *yuaA*-Spinach2 biosensor in tandem with the overexpression of *dacA* or *gdpP* (c-di-AMP encoding and degradation enzymes) on pRMC2 and pCN52 plasmid backbones with aTc as an inducer.

Data created from growth assays that used TSB as a growth media reveal several interesting facets of aerobic growth for *S. aureus.* Firstly, the *yuaA*-Spinach2 biosensor, with either *rpSJ* or *rpOD* promoters, works as a viable option for detecting c-di-AMP levels during *S. aureus* growth using a filter-based detection method because we do observe slightly higher fluorescence when comparing the *yuaA*-Spinach2 reporter to the control tRNA-Spinach2.

Despite significant data from the filter-based detection method, when comparing the Spinach2 biosensor to a control in batch cultures, fluorescence values are quite low overall. In the future, another method of fluorescence detection may be necessary to increase assay sensitivity, specifically flow cytometry, which was initially used with the *yuaA*-Spinach2 reporter in *L. monocytogenes*[7]. The use of flow cytometry has exploded over the years, and is the most viable option for further experiments due to more numerous parameters that allow for more intricate wavelength manipulation on large cell populations[9]. Flow cytometry would allow *yuaA*-Spinach2 fluorescence to be calculated on a *per cell* basis. In the current 96 well plate method of fluorescence detection, we are reading the average fluorescence of a large population of cells and must normalize to account for the number of cells by dividing OD_{650} as a proxy for cell numbers. However, because fluorescence values are so low, this normalization method may be skewing results; for example, dividing by smaller OD_{650} for cultures that grow more slowly can make fluorescence values appear relatively higher than they actually are.

Furthermore, the Biotek HTX Synergy microplate reader uses a filter-based method for detection of fluorescence; because of this its parameters can only be manipulated to a certain degree. For example, the Biotek's excitation and emission wavelengths for green fluorescence with our current filters are 485nm and 528nm, whereas according to Lenhart et al, 2020, Spinach2 bound to DFHBI has peak excitation and emission wavelengths at 447nm and 501nm. Further experiments could utilize flow cytometry with wavelengths closer to the DFHBI peaks.

Additionally, Spinach2 relies on DFHBI uptake by bacterial cells for fluorescence, which makes it susceptible to potentially variable uptake. By detecting fluorescence on a per cell basis with flow cytometry, we could eliminate any possible effects of variable DFHBI uptake within a batch culture. As explained by the colony growth inside the 96 well plate during use with the Biotek reader, fluorescence readings decrease over time because the colony growth interferes with OD_{650} readings. This ultimately impacts the analysis of the data overall. Flow cytometry would allow for real-time, kinetic, and *in vivo* detection of c-di-AMP with multi-faceted data analysis[9].

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