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In Vivo Detection of Cyclic-di-AMP in *Staphylococcus aureus*

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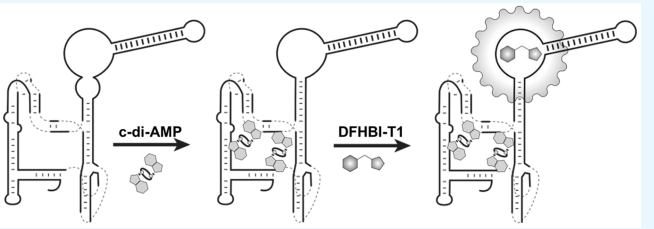
ABSTRACT: Cyclic-di-AMP (CDA) is a signaling molecule that controls various cellular functions including antibiotic tolerance and osmoregulation in *Staphylococcus aureus* (*S. aureus*). In this study, we developed a novel biosensor (*bsuO* P6-4) for in vivo detection of CDA in *S. aureus*. The fluorescent biosensor is based on a natural CDA riboswitch from *Bacillus subtilis* connected at its P6 stem to the dye-binding aptamer Spinach. Our study showed that *bsuO* P6-4 could detect a wide concentration range of CDA in both laboratory and clinical strains, making it suitable for use in both basic and clinical research applications.



1. INTRODUCTION

Cyclic-di-AMP (CDA) is a newly discovered second messenger, which is present in bacteria belonging to the phyla firmicutes and actinobacteria.[1] Recent studies have demonstrated that CDA plays important roles in regulating vital biological processes such as DNA repair, ion homeostasis, and central carbon metabolism among others.[2−5] In addition, CDA has also been implicated in controlling processes that are important for bacterial pathogenesis such as biofilm formation, antibiotic tolerance, and virulence.[6−10] CDA mediates its function through binding to its cognate effectors (i.e., proteins and riboswitches) and thereby modifying their function through allosteric changes and/or through altered gene expression. Thus, maintaining the correct concentration of CDA in bacterial cells is critical not only to retaining cellular homeostasis but also to responding to changing environmental needs. This is attained by controlling CDA's synthesis or degradation machinery(s) that is present in bacterial cells.

In *Staphylococcus aureus* (*S. aureus*) CDA synthesis and degradation are mediated by DacA (diadenylate cyclase) and GdpP (the primary CDA phosphodiesterase), respectively.[1] Recent studies have highlighted that increased CDA concentrations promote tolerance to β-lactam antibiotics and allow cell wall restructuring.[8,11] Furthermore, a growing number of contemporary clinical surveillance studies have identified mutations in *gdpP* among β-lactam resistant or nonsusceptible natural isolates of *S. aureus*.[12−15] Since many of these mutations have been either shown[8] or are predicted to attenuate the function of GdpP, causing increased CDA concentrations in cells, these findings underscored the clinical importance of CDA and highlighted the importance of accurate determination of its concentrations for both basic and clinical research settings.

2. RESULTS AND DISCUSSION

Detection and quantification of CDA are typically carried out either through HPLC/MS, indirect ELISA, or dye intercalation assay.[16−18] These assays determine CDA's abundance in a static manner, i.e., in samples containing bacterial cell lysates. Of these, HPLC/MS despite being a gold standard in the quantification of small molecules such as CDA requires expensive technical infrastructure and operational expertise. The operation of ELISA is relatively easy but requires expertise in protein purification. In this study, we present a novel RNA-based CDA biosensor (*bsuO* P6-4) (Figure 1A), which can determine its concentration in live cells through flow cytometric analysis. The RNA-based biosensor offers highly specific and sensitive detection of CDA by employing as the binding agent of natural riboswitches discovered to regulate bacterial genes in response to CDA.[19] Riboswitch-based reagents have previously been shown to have comparable to or better affinity and selectivity than commercial antibodies for small molecule antigens.[20] The fluorescent read-out is achieved by the riboswitch controlling the folding of an appended dye-binding sequence, Spinach, such that CDA binding to the riboswitch permits Spinach to bind and activate the fluorescence of the dye DFHBI-1T (Figure 1B).

bsuO P6-4 is a second-generation CDA biosensor with improved CDA affinity and signal-to-noise ratio compared to those of its predecessor, *yuaA* P1-4.[21] This improvement was

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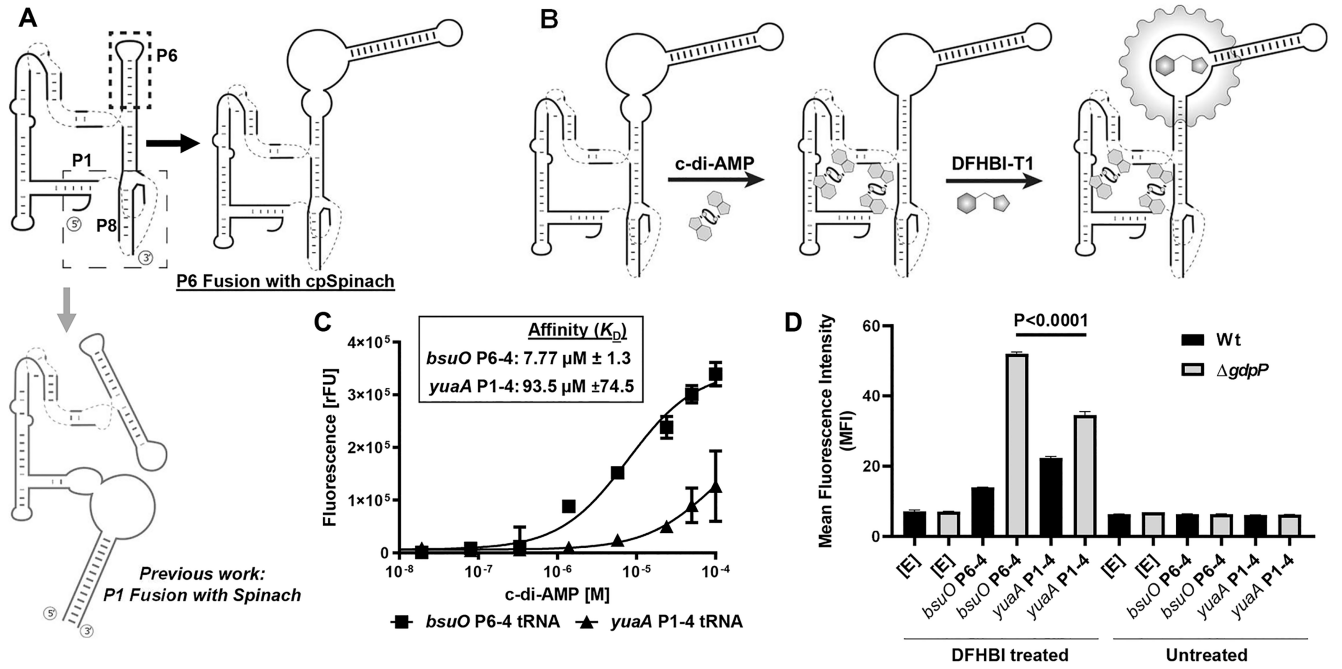

Figure 1. Construction of the cyclic-di-AMP biosensor*bsuO* P6-4 and its ability to detect cyclic-di-AMP. (A and B) Schematic representation of the creation and function of *bsuO* P6-4. (C and D) Comparison of in vitro and in vivo detection of CDA respectively by *bsuO* P6-4 and *yuaA* P1-4.

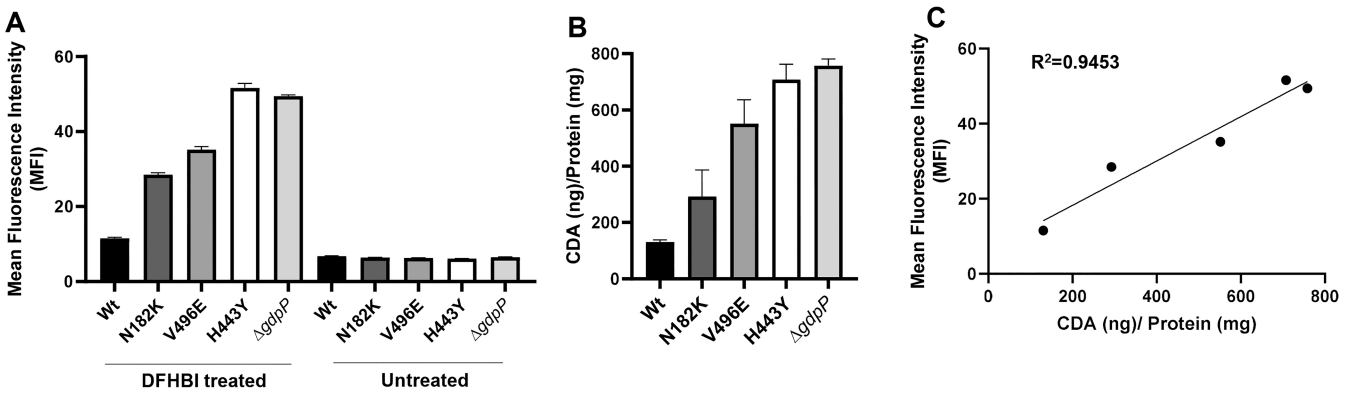

Figure 2. Ability of*bsuO* P6-4 in detecting varying concentrations of cyclic-di-AMP in isogenic strains of *S. aureus*. (A and B) Detection of CDA in wild-type and isogenic strains of *S. aureus* which carried GdpP loss of function mutations using flow cytometry and ELISA assay, respectively. (C) Correlation of signals obtained in panels A and B.

achieved by fusing the pro-fluorescent dye-binding RNA aptamer (Spinach) to the P6 stem instead of the P1 stem (as in *yuaA* P1-4) of the natural CDA binding riboswitch sequence present in the upstream region of *ydaO*/*yuaA* genes in *Bacillus subtilis* (*B. subtilis*)[19] (Figure 1A). This rational design of *bsuO* P6-4 restored the pseudoknot interaction between the P1 and P8 stems, which acts as a native stabilizer of the *ydaO*/*yuaA* riboswitch structure.[22−24] Additionally, a modified fluorescent dye binding module, coined as cpSpinach2,[20] that was circularly permutized to accept a transducer stem was used for the construction of *bsuO* P6-4 (Figure 1A). Thus, *bsuO* P6-4 consists of two components, the CDA-binding *ydaO*/*yuaA* module, and the pro-fluorescent, DFHBI-binding cpSpinach2 module. Binding of CDA to *bsuO* P6-4 enabled appropriate folding of the cpSpinach2 module, allowing DFHBI binding and production of a fluorescence signal (Figure 1B) and thereby enabling detection of CDA. The specificity of the CDA-binding *ydaO*/*yuaA* module to particularly detect CDA has been shown previously.[21]

An in vitro fluorescence assay testing the CDA biosensors revealed a > 10-fold higher affinity of *bsuO* P6-4 compared to its predecessor *yuaA* P1-4 (Figure 1C). In preparation for in vivo experiments, a tRNA scaffold was added to flank the 5′ and 3′ ends of the biosensors for increased RNA half-life (Supporting Information Table S1).[25] The resultant biosensors were cloned into a constitutive expression vector and transformed into a wild-type (Wt) *S. aureus* and its isogenic Δ*gdpP* strain. While both biosensors were able to report the higher level of CDA that is characteristic of a Δ*gdpP* strain,[8] *bsuO* P6-4 showed significantly enhanced fluorescence compared to that of *yuaA* P1-4. More importantly, *bsuO* P6-4 compared to *yuaA* P1-4 displayed a higher dynamic range of signal (3.73X vs 1.55X) between Wt and Δ*gdpP* strains making it amenable for detection of a wider concentration range of

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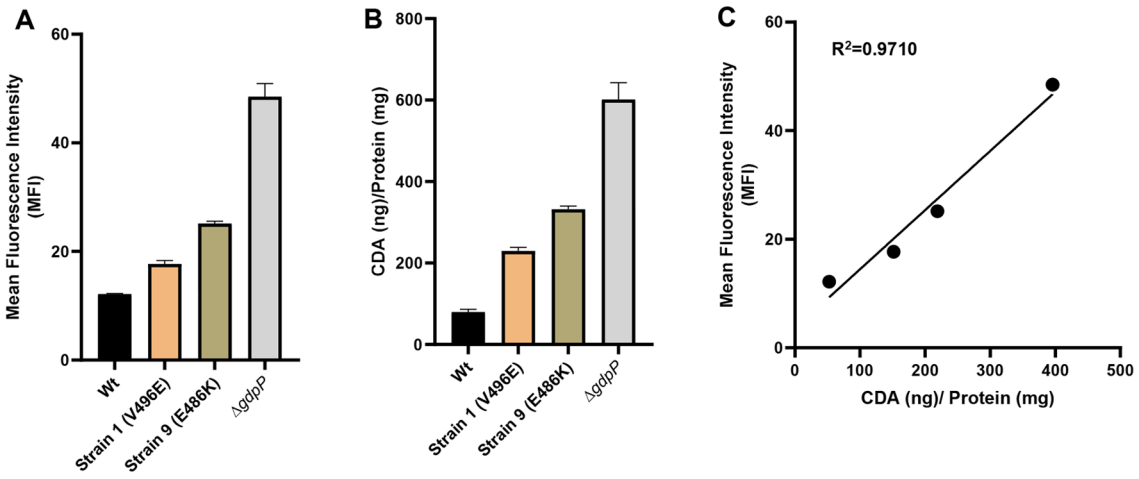

Figure 3. Ability of*bsuO* P6-4 in detecting varying concentrations of cyclic-di-AMP in clinical strains of*S. aureus*. (A and B) Detection of CDA in wild-type and clinical strains of *S. aureus* which carried GdpP loss of function mutations using flow cytometry and ELISA assay, respectively. (C) Correlation of signals obtained in panels A and B.

CDA (Figure 1D). The reason why *yuaA* P1-4 exhibits higher background fluorescence than *bsuO* P6-4 in vivo is unknown.

Next, we sought to determine whether *bsuO* P6-4 could report different concentrations of CDA in the cells. For this purpose, isogenic *gdpP* point mutants were created that displayed varying degrees of GdpP's loss of function that were identified in our previous study.[8] As shown in Figure 2, *bsuO* P6-4 was able to detect differing CDA concentrations in the isogenic strains (Figure 2A), which correlated well (R^2= 0.9453) with the results independently obtained through ELISA assay among the identical strains (Figure 2B,C). In addition to the isogenic strains, *bsuO* P6-4 was also able to determine different CDA concentrations in clinical isolates (Figure 3), which suggested that it could be used in both laboratory and clinical strains.

In summary, we have developed a novel *bsuO* P6-4 biosensor that is effective in determining different CDA concentrations in live *S. aureus* cells through flow cytometry. The plasmid harboring *bsuO* P6-4 can be transformed into both laboratory and clinical *S. aureus* isolates for reporting CDA concentration. This biosensor-based approach could be used in flux detection of CDA concentrations in future studies.

3. METHODS

3.1. Bacterial Strains, Growth Media, and Growth Conditions. Bacterial strains used in this study are shown in Table S2. Bacteria were grown in trypticase soy broth (TSB) at 37 °C with shaking at 180 rpm or on trypticase soy agar (TSA) plates at 37 °C. Strains containing plasmids pTX_{Δ}, *pJB*38, and *pET*28b(+) were grown in tetracycline (12.5 μg/mL), chloramphenicol (10 μg/mL), and kanamycin (50 μg/mL), respectively, as selection markers. The molecular cloning experiments were carried out using *S. aureus* RN4220 strains. Lists of plasmids and primers used in this study are shown in Tables S3 and S4, respectively. Sequence fidelity of all of the mutants and constructs was validated using analytical PCR and Sanger sequencing.

3.2. Construction of Mutants. Construction of mutants with *gdpP* point mutations (i.e., N182K, V496E, and H443Y) was carried out using plasmid *pJB*38 as described previously.[8,26] Briefly, a splice-overlap PCRs of the 1 kb up- and downstream genomic region surrounding the *gdpP* mutations were amplified using either primers gdpP-SacI1-Nterm-F and gdpP-XmaI-Nterm-R or gdpP-SacI-Cterm-F and gdpP-XmaI-Cterm-R and with the primers labeled with the respective point mutations (Table S4). The resulting PCR product was digested with *SacI* and *XmaI* and ligated into *pJB*38. The plasmid was transformed into SF8300, and the standard allelic replacement procedure was carried out as shown before.[26] The mutants were sequence-verified to confirm the *gdpP* point mutations.

3.3. Construction of *bsuO* P6-4, In Vitro Transcription, In Vitro CDA Affinity Determination, and Cloning into pTX_{Δ}. *3.3.1. Construction of bsuO P6-4.* *bsuO* P6-4 was constructed by Golden Gate cloning. Briefly, the *Bacillus subtilis ydaO* (*bsuO*) sequence cloned into a *TOPO PCR 2.1* vector was PCR-amplified "around-the-horn" with overhang primers BsuO-P6-Fwd and BsuO-P6-Rev to add *BsaI* restriction sites at the P6 stem sequence. A circularly permutized Spinach2 sequence (cpSpinach2[20] was amplified with overhang primers BsuO-P6-4-Spin-Fwd and BsuO-P6-4-Spin-Rev adding sticky ends and *BsaI* restriction sites. After Golden Gate assembly (restriction with *BsaI*-HF, 50 °C, 1 h, and ligation with T4 ligase, 30 min, 24 °C; both NEB), constructs were transformed into TOP10 chemically competent *Escherichia coli* (*E. coli*; Invitrogen) and clones recovered and sequenced. To assemble tRNA construct via Gibson assembly, *pET-31b tRNA*[27] was amplified with primers adding homology sites to *bsuO* (BsuO-tRNA-GA-Fwd and BsuO-tRNA-GA-Rev), and *bsuO* P6-4 was amplified with primers adding homology sites to the tRNA scaffold (tRNA-BsuO-GA-Fwd and tRNA-BsuO-GA-Rev). After Gibson assembly (NEB), constructs were transformed into TOP10 chemically competent *E. coli* (Invitrogen) and clones recovered and sequenced.

3.3.2. In Vitro Transcription. Biosensor RNA was transcribed using T7 RNA polymerase (NEB) and previously published protocols.[28] In brief, a DNA transcription template was produced by PCR amplification with primers T7-Fwd and tRNA-Rev from a sequence-confirmed plasmid PCR template. Transcribed products were purified by PAGE, quantified after thermal hydrolysis,[29] and stored at −80 °C.

3.3.3. CDA Affinity Determination. Apparent affinity (K_d) was determined by fluorescence response in a 384-well plate

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reader assay, as published previously. Biosensor RNA (30 nM) and DFHBI (10 μM) were incubated reaction buffer (40 mM HEPES, pH 7.5, 125 mM KCl, 3 mM $MgCl_2$) with increasing concentrations of CDA (Biolog) at 37 °C. Upon reaching equilibrium after 3 h, fluorescence (excitation, 448 nm; emission, 508 nm) was determined using a SpectraMax Paradigm plate reader and analyzed using GraphPad Prism 9.

3.3.4. Cloning into pTX_Δ. Biosensor constructs were PCR-amplified using primers Spinach-BamHI-F and Spinach-MluI-R (Table S4), digested with *BamHI* and *MluI* and were ligated into the pTX_Δ plasmid. The resulting plasmid was transformed into RN4220 via electroporation and was sequence-verified. The plasmid was transduced into the final strains using the phage Φ11.

3.4. Quantification of c-di-AMP Levels in *S. aureus* Using Flow Cytometer. Quantification of CDA was carried out as previously described in Kellenberger et al.[21] with slight modifications. *S. aureus* strains containing pTX_Δ + *busO* P6-4, pTX_Δ + *yuaA* P1-4, or an empty pTX_Δ vector were inoculated using a 1 μL loop from a plate into culture tubes containing 4 mL of TSB media containing tetracycline (12.5 μg/mL) and shaken at 37 °C for 18−22 h at 180 rpm. 500 OD_{600} worth of bacterial cultures were harvested and pelleted by centrifugation at 6000*g* for 5 min. The cell pellets were washed with 1× PBS and then resuspended in 100 μL of TSB media containing tetracycline (12.5 μg/mL) and with or without DFHBI-1T (200 μM). The resuspended bacterial samples were incubated at 37 °C for 1 h in the dark. After incubation, 40 μL of bacteria was diluted into 1 mL of 1× PBS, 200 μL of which was dispensed into a 96-well plate in triplicate, and analyzed with a Guava easyCyte Flow cytometer (Luminex, USA) (parameters: 30000 events; excitation, blue laser (488 nm); emission, green channel (512−530 nm); fluidics, slow; cutoff, 0). The data were analyzed using InCyte from guavaSoft 4.0 software, and the mean fluorescent intensity from the GFP channel was calculated for each sample.

3.5. Quantification of Cyclic-di-AMP Levels in *S. aureus* Using Competitive ELISA Assay. Determination of CDA levels using Competitive ELISA assay was carried out as described in Underwood et al.[17] with slight modifications.

3.5.1. CabP Cloning and Protein Purification. The *cabP* gene was PCR-amplified from *Streptococcus pneumoniae* D39 genomic DNA using primers cabP-NdeI-F and cabP-*Hind*III-R and cloned into the *pET28b(+)* vector using *NdeI* and *HindIII* restriction enzymes. The resulting plasmid was transformed into DH5α via heat shock and sequence-verified for accuracy. The resulting *pET28b(+)* + *cabP* was transformed into *E. coli* BL21(DE3). CabP protein was overexpressed and purified as described in Bai et al.[30]

3.5.2. CDA Extraction from S. aureus. Bacteria were inoculated using a 1 μL loop from a plate into culture tubes containing 4 mL of TSB media and shaken at 37 °C for 18−22 h at 180 rpm. 30 OD_{600} of bacterial culture was harvested, pelleted at 2739*g* for 10 min, and washed with 1× PBS. Cell pellets were resuspended with 600 μL of 50 mM Tris HCl (pH 8.0) and lysed using a FastPrep-24 set to 6.5 m/s and 45 s for 4 cycles (MP Biomedicals). Lysed cells were centrifuged at 17000*g* for 10 min at 4 °C, and supernatants were collected. An aliquot of the total lysate was saved and later used for protein estimation using Pierce BCA Protein Assay kit (Thermo Fisher). Each sample's CDA concentration (ng/mL) was normalized with respect to protein content (mg/mL). Supernatants were boiled at 95 °C for 10 min, allowed to rest to room temperature for 10 min, and centrifuged at 17000*g* for 10 min. The boiled supernatants were collected, diluted in half with 50 mM Tris HCl (pH 8.0), and used for Competitive ELISA assay for CDA quantification.

3.5.3. Competitive ELISA Assay. CDA quantification carried out as described in Underwood et al.[17] HRP-conjugated streptavidin (Thermo Scientific) diluted to a 1:5000 dilution in PBS was used after incubation of samples on the ELISA plate.

3.6. Statistical Analysis. Statistical analysis was carried out using GraphPad Prism 8.1.1. Comparisons and significance were determined using Student's *t* test. Each experiment was repeated at least twice to ensure reproducibility.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.2c04538.

(Table S1) Biosensor sequences, (Table S2) list of strains, (Table S3) list of plasmids, and (Table S4) list of primers used in this study (PDF)

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Author Contributions

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Notes

The authors declare no competing financial interest.

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