In-vivo detection of cyclic-di-AMP in *Staphylococcus aureus*

Running Title: RNA biosensor based detection of cyclic-di-AMP in *S. 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*. In this study, we developed a novel biosensor (*bsuO* P6-4) for *in-vivo* detection of CDA in *S. aureus*. 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.

KEYWORDS: Cyclic-di-AMP, biosensors, *Staphylococcus aureus*

24 **Main text**

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

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

47 Detection and quantification of CDA are typically carried out either through HPLC/MS,
48 indirect ELISA, or dye intercalation assay (16-18). These assays determine CDA's
49 abundance in a static manner, i.e. in samples containing bacterial cell lysates. Of these,
50 HPLC/MS despite being a gold standard in the quantification of small molecules such as
51 CDA requires expensive technical infrastructure and operational expertise. The operation
52 of ELISA is relatively easy but requires expertise in protein purification. In this study, we
53 present a novel RNA-based CDA biosensor (*bsuO* P6-4), which can determine its
54 concentration in live cells through flow cytometric analysis.
55 *bsuO* P6-4 is a second-generation CDA biosensor with improved CDA affinity and signal-
56 to-noise ratio than its predecessor, *yuaA* P1-4 (19). This improvement was achieved by
57 fusing the pro-fluorescent dye-binding RNA aptamer (Spinach) to the P6 stem instead of
58 the P1 stem (as in *yuaA* P1-4) of the natural CDA binding riboswitch sequence present in
59 the upstream region of *ydaO*/*yuaA* genes in *Bacillus subtilis* (20) (Fig.1A). This rational
60 design of *bsuO* P6-4 restored the pseudoknot interaction between the P1 and P8 stems,
61 which acts as a native stabilizer of the *ydaO*/*yuaA* riboswitch structure (21-23).
62 Additionally, a modified fluorescent dye binding module, coined as cpSpinach2 (24) that
63 was circularly permutized to accept a transducer stem was used for the construction of
64 *bsuO* P6-4 (Fig.1A). Thus, *bsuO* P6-4 consists of two components, the CDA-binding
65 *yda*O/*yua*A module, and the pro-fluorescent, DFHBI-binding cpSpinach2 module. Binding
66 of CDA to *bsuO* P6-4 enabled appropriate folding of the cpSpinach2 module, allowing
67 DFHBI binding and production of a fluorescence signal (Fig.1B) and thereby enabling
68 detection of CDA.

69 An *in-vitro* fluorescence assay testing the CDA biosensors revealed a >10-fold higher
70 affinity of *bsuO* P6-4 compared to its predecessor *yuaA* P1-4 (Fig.1C). In preparation for
71 *in-vivo* experiments, a tRNA scaffold was added to flank the 5' and 3' ends of the
72 biosensors for increased RNA half-life (Table S1) (25). The resultant biosensors were
73 cloned into a constitutive expression vector and transformed into a wild-type (Wt) *S.*
74 *aureus* and its isogenic Δ*gdpP* strain. While both the biosensors were able to report the
75 higher level of CDA that is characteristic of a Δ*gdpP* strain (8), *bsuO* P6-4 showed
76 significantly enhanced fluorescence compared to that of *yuaA* P1-4. More importantly,
77 *bsuO* P6-4 compared to *yuaA* P1-4 displayed a higher dynamic range of signal (3.73X vs
78 1.55X) between Wt and Δ*gdpP* strains making it amenable for detection of a wider
79 concentration range of CDA (Fig.1D). The reason why *yuaA* P1-4 exhibits higher
80 background fluorescence than *bsuO* P6-4 *in vivo* is unknown.
81 Next, we sought to determine whether *bsuO* P6-4 could report different concentrations of
82 CDA in the cells. For this purpose, isogenic *gdpP* point mutants were created that
83 displayed varying degrees of GdpP's loss of function that were identified in our previous
84 study (8). As shown in figure 2, *bsuO* P6-4 was able to detect differing CDA
85 concentrations in the isogenic strains (Fig.2A), which correlated well (R^2=0.9453) with the
86 results independently obtained through ELISA assay among the identical strains (Fig.2B
87 & C). In addition to the isogenic strains, *bsuO* P6-4 was also able to determine different
88 CDA concentrations in clinical isolates (Fig. S1), which suggested that it could be used in
89 both laboratory and clinical strains. Our results further showed that the Wt and Δ*gdpP*
90 strains with *bsuO* P6-4 were also suitable for fluorescent microscopic analysis (Fig. S2).

However, this method was not sensitive enough to differentiate the intermediate concentrations of CDA of the other isogenic strains (data not shown).

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.

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185 **FIGURE LEGENDS**

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

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

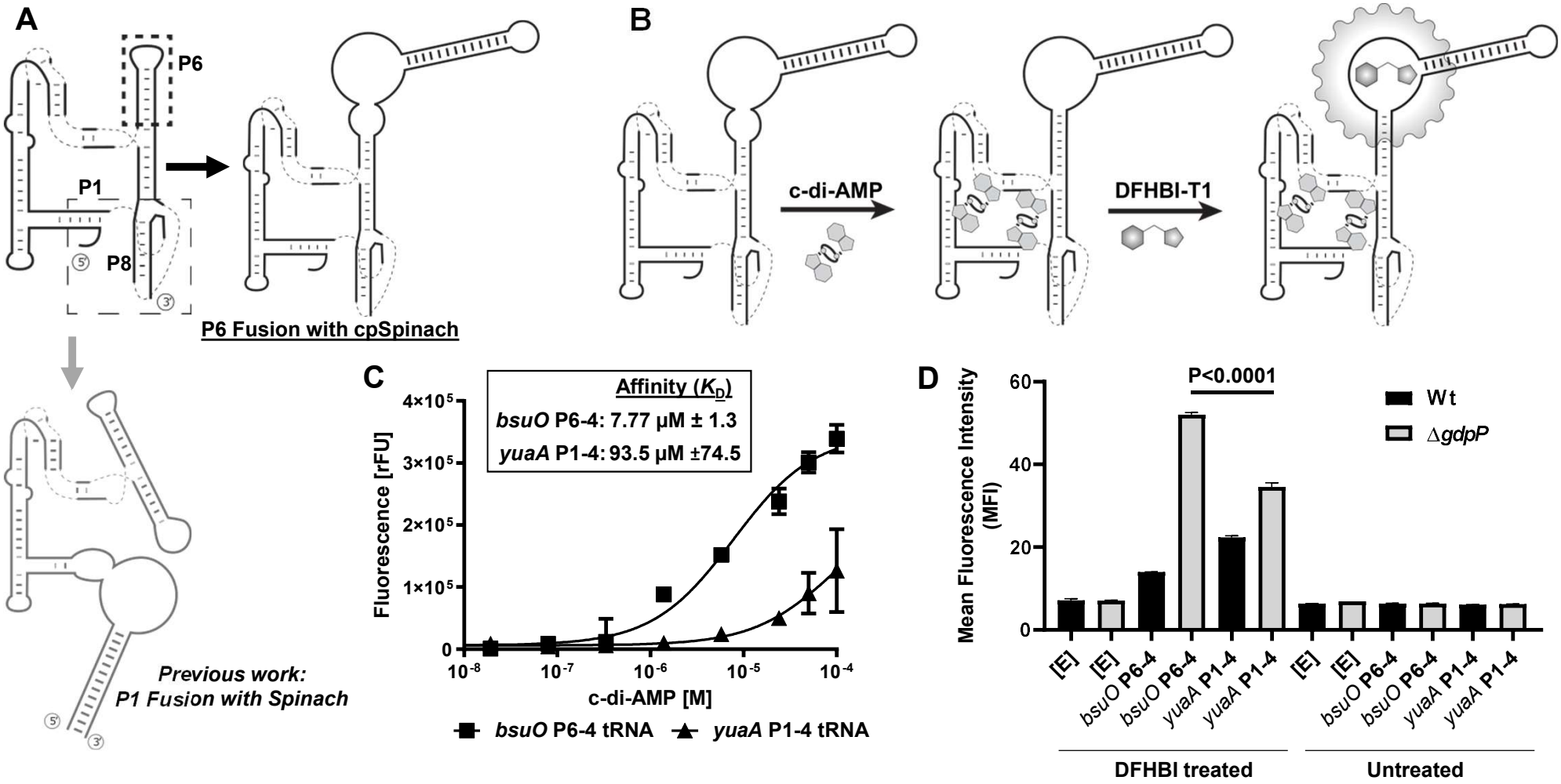

Figure 1: Construction of the cyclic-di-AMP biosensor *bsuO* P6-4 and its ability to detect cyclic-di-AMP. A) & B) Schematic representation of the creation and function of *bsuO* P6-4. C) & D) Comparison of *in-vitro* and *in-vivo* detection of CDA respectively by *bsuO* P6-4 and *yuaA* P1-4.

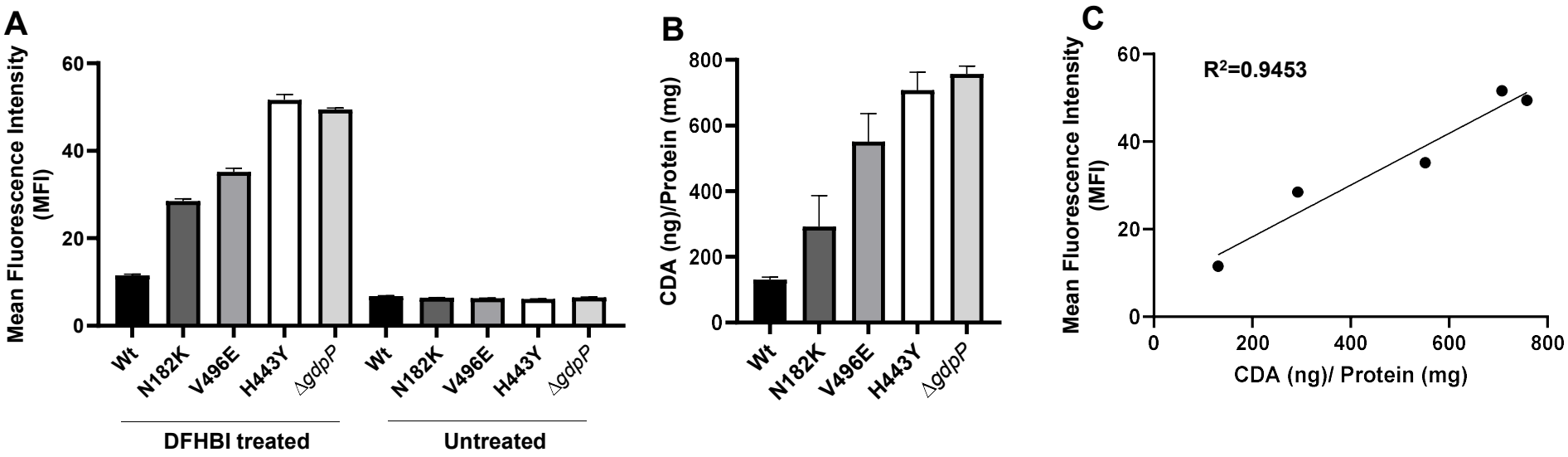

Figure 2: Ability of *bsuO* P6-4 in detecting varying concentrations of cyclic-di-AMP in *S. aureus*. A) & B) Detection of CDA in wild-type and isogenic strains of *S. aureus* which carried GdpP loss of function mutations using flow cytometry and ELISA assay respectively. C) Correlation of signals obtained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