Supporting Information for

# RNA-Based Fluorescent Biosensors for Live Cell Imaging of Second Messenger Cyclic di-AMP

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**Contents:**

## General reagents and equipment

DNA oligonucleotides were purchased as Ultramer oligos from IDT (Coraleville, IA). Cyclic and linear dinucleotides were purchased from Axxora (Farmingdale, NY) and were used without further purification. DFHBI and DFHBI-1T fluorophores were either purchased from Lucerna (New York, NY) or were synthesized following previously described protocols[1,2] and were stored as 20-40 mM stocks in DMSO at -20 °C. *In vitro* fluorescent measurements were made using a Molecular Devices SpectraMax Paradigm plate reader.

## *In vitro* transcription

Sequences of all biosensor constructs are given in Table S2. RNA was transcribed following previously reported protocols.[3,4] Briefly, a DNA template was prepared by PCR amplification of the desired sequences using primers that added a T7 promoter directly upstream of the Spinach construct. DNA templates were purified by PCR purification, then were used in a transcription reaction with T7 RNA polymerase (NEB, Ipswich, MA). RNAs were purified by denaturing polyacrylamide gel electrophoresis (PAGE), then were eluted from gel pieces and ethanol precipitated. Accurate RNA concentrations were determined using a thermal hydrolysis assay to eliminate the hypochromic effect from these structured RNAs.[5]

## *In vitro* ligand binding assays

Fluorescent ligand binding assays were performed as previously described.[3,4] In brief, reactions were prepared in a 96-well Corning Costar (Tewksbury, MA) 3915 black plate

with final buffer concentrations of 40 mM HEPES, pH 7.5, 125 mM KCl, either 3 or 10 mM $MgCl_2$ with 10 µM DFHBI and the appropriate ligand concentration. RNAs were renatured by mixing with an equal volume of binding buffer (80 mM HEPES, pH 7.5, 250 mM KCl, and either 6 or 20 mM $MgCl_2$), heating it to 70 °C for 3 min, and then cooling at ambient temperature for 5 min before adding to the reactions at a 10-fold dilution (30 nM or 100 nM final concentration). Fluorescence was monitored at 5 min intervals using an excitation of 460 nm and emission of 500 nm. Data was collected and analyzed after samples had reached equilibrium (within 0.5-2 h).

In general, assays to screen for ligand binding were carried out at 30 °C in buffer with 10 mM $MgCl_2$, which increases the sensitivity of these experiments.[3] Assays aimed at characterizing biosensor performance under physiological conditions were carried out at 37 °C in buffer with 3 mM $MgCl_2$.

### *In vitro* association and dissociation experiments

Association of the biosensors with ligand was measured following a similar protocol as described previously.[3,4] Briefly, buffer containing 40 mM HEPES, pH 7.5, 125 mM KCl, 3 mM MgCl2, 10 µM DFHBI, and the appropriate ligand was pre-warmed to 37 °C. The RNA (final concentration 100 nM) was renatured as described above and pre-warmed to 37 °C before mixing with buffer solution. Fluorescence was monitored immediately after RNA addition at 2 min intervals with an excitation of 460 nm and emission of 500 nm.

Ligand dissociation experiments were prepared by first equilibrating RNA (100 nM) and ligand (100 µM for P1-4 yuaA-Spinach2 and 10 µM for C3A Sc1-Spinach2) at 37 °C in the buffer described for association experiments. A deactivation buffer (200 µL) containing just 40 mM HEPES, pH 7.5, 125 mM KCl, 3 mM $MgCl_2$, and 10 µM DFHBI was pre-warmed in the same plate to 37 °C. Once the RNA binding reaction had equilibrated, it was diluted 100-fold into the pre-warmed deactivation buffer. Fluorescence was monitored immediately after dilution at 1 min intervals with an excitation of 460 nm and emission of 500 nm.

**Molecular cloning**

For experiments in *L. monocytogenes*, the yuaA-Spinach2 tRNA biosensor was cloned into the high copy number shuttle plasmid pAM401 or the integration plasmid pPL2. Briefly, Splicing by Overlap Extension (SOEing) PCR was used to fuse a *L. monocytogenes* small RNA promoter (Pli31)[6] or a constitutive promoter (Phyper)[7,8] with the yuaA-Spinach2 construct flanked on either side by a tRNA scaffold and a T7 terminator at the 3' end.[3,9] SOE PCR products were gel purified, digested with EagI and SalI and then ligated with either pAM401 or pPL2-Cm digested with the same restriction enzymes. For the phosphodiesterase (PDE) gene complementation study, the *L. monocytogenes* gene *pdeA* (gene ID: *lmo0052*) was truncated by PCR and cloned into KpnI/XhoI sites of the pPL2-Erm integration plasmid under control of its native promoter (Table S4).

The resulting plasmids were confirmed by sequencing and transformed into different *L. monocytogenes* strains as previously described.[10] Briefly, pAM401 or pPL2-Erm was transformed into *E. coli* strain SM10 (conjugation donor), and the resulting strains were mated with different *L. monocytogenes* recipient strains carrying a streptomycin resistance mutation. Strains and plasmids that were used in this study are listed in Table S5.

For experiments in *E. coli,* codon optimized candidate *disA*-like genes (Table S4) were synthesized as gBlocks by Integrated DNA Technologies (IDT) and cloned into NdeI/XhoI sites of pCola-duet 1 plasmid($Kan^R$). Different biosensors were cloned as previously described.[3,9] Each biosensor in pET31b was co-transformed into *E. coli* with an enzyme construct in pCOLADuet-1 or empty pCOLADuet-1 vector as a control.

**Fluorescence microscopy**

Fluorescence microscopy experiments of bacterial cultures were carried out following previous protocols.[3,9,11] Briefly, ~1 $OD_{600}$ of *L. monocytogenes* overnight culture grown in BHI media was spun down at 5000 rpm for 5 min, and resuspended in 2 mL of PBS. A 50 µL aliquot of resuspended culture was then plated on poly-L-lysine coated 96 well glass-bottom dishes (MatTek) to adhere the cells for one hour at 37 °C. Adherent bacteria were washed three times with PBS to remove unbound cells and then incubated with 1 µM FM 4-64 (Life Technologies) and 200 µM DFHBI for 30 min at 37 °C. Fluorescent images were taken through a 100x oil objective mounted on a Olympus

IX71 microscope using DAPI and TRITC filters. All images were taken with same exposure settings and analyzed with Image J.

**Flow cytometry**

Flow cytometry experiments were carried out by pelleting ~1 $OD_{600}$ of overnight *L. monocytogenes* culture that then were washed twice with 1x PBS and resuspended in 1 mL PBS. Then, 10 µL of bacterial culture was mixed with 100 µL of 100 µM DFHBI or DFHBI-1T in 1x PBS in a polystyrene FACS tube. Samples were protected from light and incubated at 37 °C for 30 min before flow cytometry analysis. Data was collected by LSR Fortessa (BD Biosciences) and analyzed using FlowJo software. For each sample at least 50,000 bacteria were collected by size and singlet gates (FSC-H/FSC-A). Then, mean fluorescent intensity from the FITC channel was calculated for each sample.

**Cell extraction and HPLC-MS analysis**

Cyclic dinucleotides were extracted following previously described protocols.[12,13] Briefly, 100 mL *E. coli* expressing diadenylate cyclase was pelleted at 4700 rpm for 10 minutes. Cell pellets were resuspended in 1400 µL extraction buffer (40% methanol, 40% acetonitrile, 20% $ddH_2O$). The solution was incubated at ambient temperature for 20 min then lysed cells were centrifuged at 4 °C for 20 min at 13,200 rpm and the supernatant was carefully removed and stored on ice. This extraction was repeated twice more with 700 µL extraction buffer. Combined supernatants were purified through a 10 kDa MWCO Amicon Ultra-4 Protein Concentrator (Millipore). Extracts were analyzed by HPLC-MS exactly as described previously.[13]

## Supplementary Results

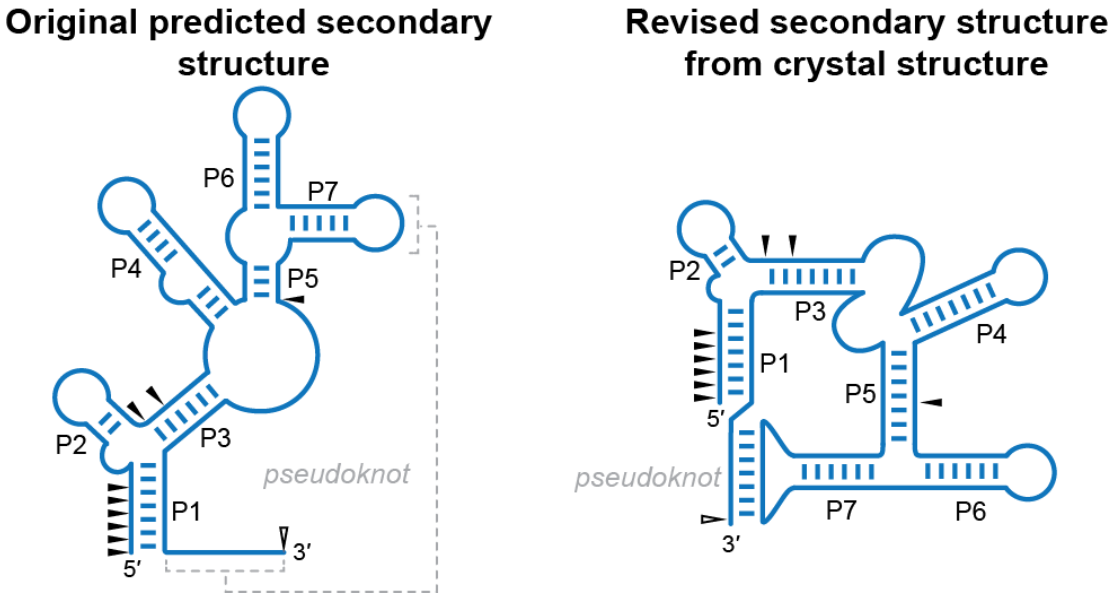



**Figure S1.** Schematic of yuaA fusion sites to Spinach2. A model of the yuaA secondary structure is shown both before and after the crystal structures of three ydaO class aptamers were solved. Black-filled arrowheads indicate base pair positions at which the Spinach2 aptamer was attached in ydaO or yuaA-Spinach2 fusions. The open arrowheads depict constructs that contained the entire aptamer sequence, including the 3′ end of the pseudoknot.

Full-length yuaA sequence:
ucucuuuuaccgcuuaaucaaaacacgaacgggggaaccaacgauuggcuguuuuauuaacagcc
uuggggugaaucuuacuaaguaagaggggguacucugaaucccuaauccgacagcuaaccucgu
aggcguauacagagaggagg

Yellow = P1 stem
Green = P3 stem
Purple = P5 stem

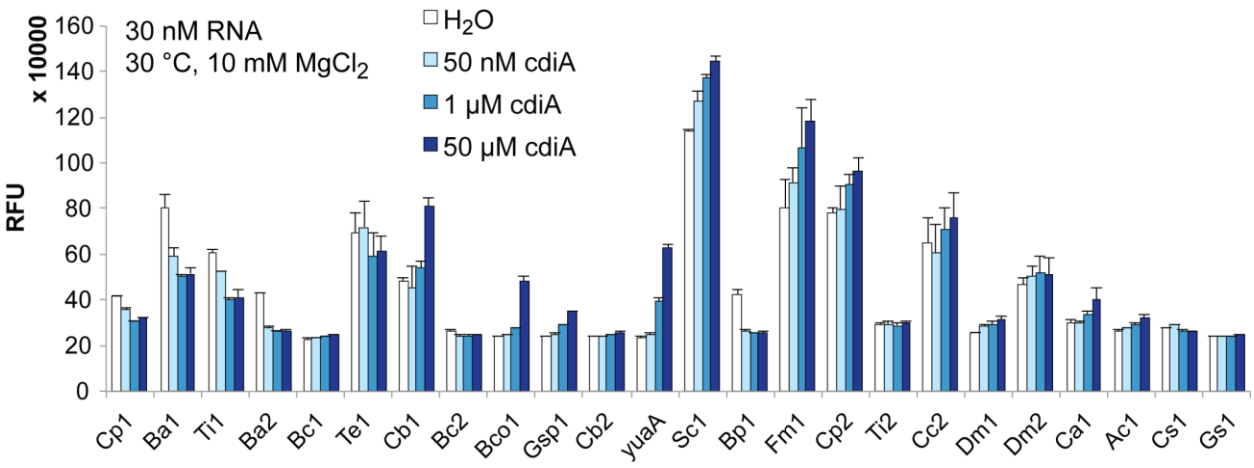
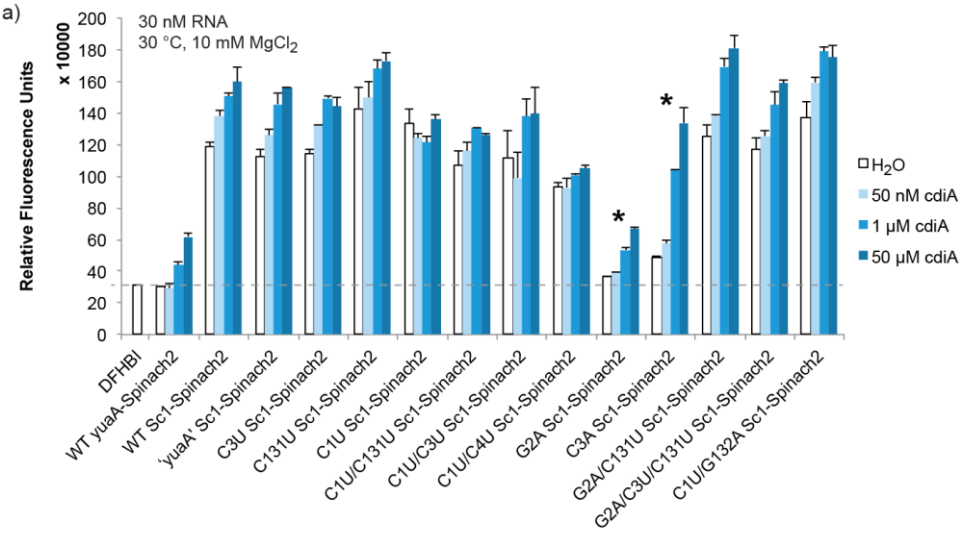



**Figure S2.** Fluorescence activation screen of additional riboswitch phylogenetic variants attached using P1-4, which led to identification of Sc1-Spinach2, a candidate biosensor with high ligand sensitivity. Error bars represent standard deviation between two independent replicates. See Table S2 for sequences.

Sc1 was chosen for optimization because it was the brightest overall sensor candidate with the highest apparent affinity from the phylogenetic screen. We have not tested variants of Fm1 or Cp2.

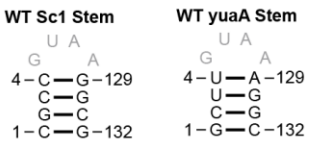
a)




b)

| Sequence | P1 stem | Stem Stability (kcal/mol) | NL Fluorescence (RFU) |
|---|---|---|---|
| WT yuaA-Spinach2 | gcuu guaa aggc | -1.9 | 301881 |
| WT Sc1-Spinach2 | cgcc guaa ggcg | -7.7 | 1189934 |
| 'yuaA' Sc1-Spinach2 | gcuu guaa aggc | -1.9 | 1121903 |
| C3U Sc1-Spinach2 | cguc guaa ggcg | -5 | 1147130 |
| C131U Sc1-Spinach2 | cgcc guaa ggug | -5.8 | 1429910 |
| C1U Sc1-Spinach2 | ugcc guaa ggcg | -6.7 | 1332865 |
| C1U/C131U Sc1-Spinach2 | ugcc guaa ggug | -4.8 | 1065923 |
| C1U/C3U Sc1-Spinach2 | uguc guaa ggcg | -3.5 | 1118343 |
| C1U/C4U Sc1-Spinach2 | ugcu guaa ggcg | -1.6 | 935145 |
| G2A Sc1-Spinach2 | cacc guaa ggcg | -2.8 | 366104 |
| C3A Sc1-Spinach2 | cgac guaa ggcg | 0.5 | 488447 |
| G2A/C131U Sc1-Spinach2 | cacc guaa ggug | -6.2 | 1253840 |
| G2A/C3U/C131U Sc1-Spinach2 | cauc guaa ggug | -3.6 | 1170235 |
| C1U/G132A Sc1-Spinach2 | ugcc guaa ggca | -7 | 1372988 |

c)




d)

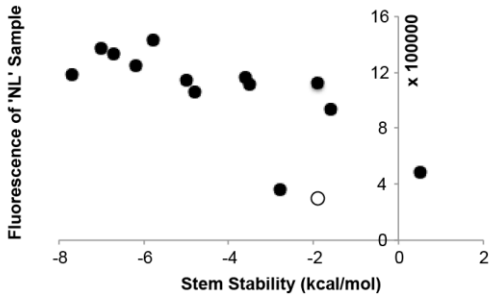



**Figure S3.** Mutation of the Sc1-Spinach2 transducer stem reveals two constructs with lower background fluorescence (starred). (a) *In vitro* fluorescence screen of P1-4 Sc1-Spinach2 mutants. RNA (30 nM) was incubated with DFHBI (10 µM) and different concentrations of cdiA at 30 °C in buffer containing 10 mM $MgCl_2$. The horizontal dashed line represents the fluorescence background of DFHBI alone in buffer. Error bars represent the standard deviation between two independent replicates. (b) Analysis of transducer stem stability via mfold using default parameters. The stabilities of the four base-pair stems were calculated for the given stem with a GUAA tetraloop inserted as shown in part (c). (d) Graphical analysis of the results presented in (b). The open circle represents the WT yuaA-Spinach2 aptamer.

While the WT Sc1-Spinach2 aptamer, which contains four G-C Watson-Crick base pairs in the transducer stem (Figure S1b), displayed 4-fold higher background fluorescence than WT yuaA-Spinach2 in the absence of ligand, disruption of one of these base pairs with either a G2A or a C3A mutation of the transducer stem reduced the background fluorescence to within 1.2 or 1.6-fold the background of the yuaA-Spinach2 aptamer, respectively. However, grafting of the P1-4 yuaA stem in place of the Sc1 stem ('yuaA' Sc1-Spinach2) and other disruptive mutations of the P1-4 Sc1 stem did not reduce the background fluorescence to within 2-fold of the fluorescence of DFHBI in solution. The C3A Sc1-Spinach2 biosensor was carried forward in these studies due to its greater fluorescence activation compared to G2A (2.7 versus 1.8-fold, respectively).

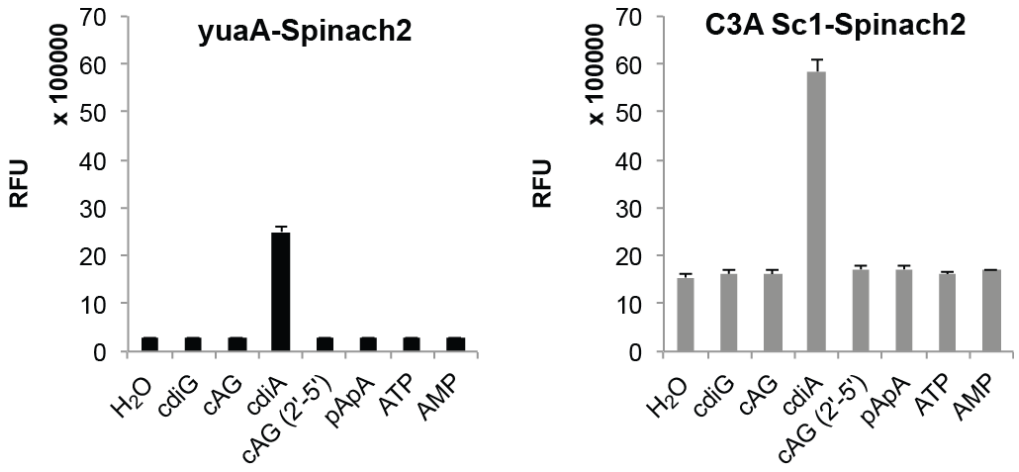



**Figure S4.** *In vitro* selectivity of the cdiA biosensors. The yuaA- and C3A Sc1-Spinach2 biosensors (100 nM) were incubated with the ligands shown (100 µM) and DFHBI (10 µM). Error bars represent the standard deviation between three independent experiments.

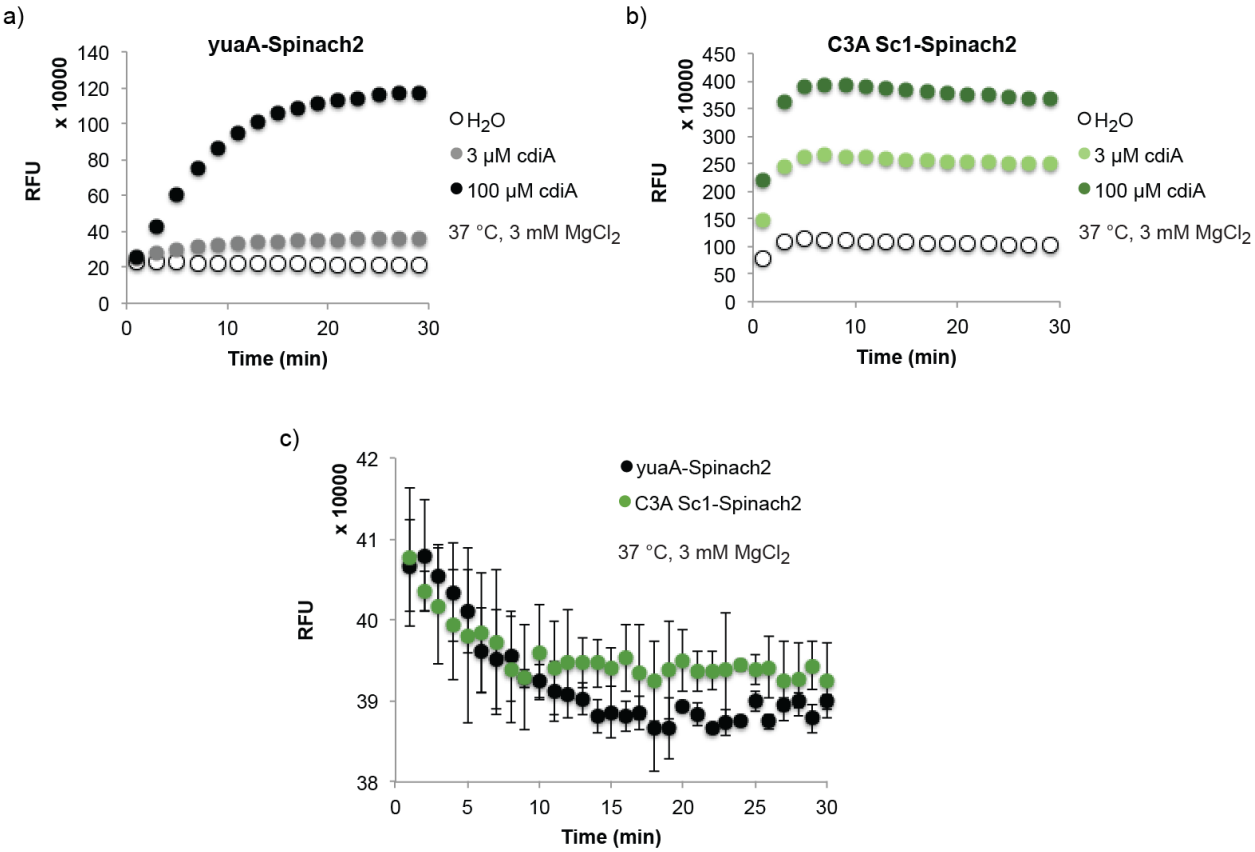



**Figure S5.** Association and dissociation kinetics of the yuaA-Spinach2 and C3A Sc1-Spinach2 biosensors. (a) and (b) Activation of the yuaA-Spinach2 and C3A Sc1-Spinach2 sensors, respectively. DFHBI (10 µM) and varied cdiA concentrations or $H_2O$ were pre-equilibrated at 37 °C in buffer containing 3 mM $MgCl_2$ before mixing with RNA (100 nM) pre-warmed to 37 °C. Fluorescence measurements were recorded immediately after addition of RNA. Data are representatives of one experiment that has been repeated twice. (c) Deactivation of the yuaA-Spinach2 and C3A Sc1-Spinach2 biosensors. RNA (100 nM) that had been pre-equilibrated with DFHBI (10 µM) and cdiA (100 µM for yuaA-Spinach2, 10 µM for C3A Sc1-Spinach2) at 37 °C in buffer containing 3 mM $MgCl_2$ was diluted 100-fold into buffer containing DFHBI (10 µM) without cdiA or RNA. Fluorescence was monitored immediately after dilution. Error bars represent the standard deviation of duplicate samples.

Full activation of the P1-4 yuaA-Spinach2 and C3A Sc1-Spinach2 biosensors occurs at ~30 min and 7 min, respectively. Deactivation of the sensors occurs within 15 and 9 min for the yuaA-Spinach2 and C3A Sc1-Spinach2 sensors, respectively. As observed previously, 75% activation and deactivation occurs in ~half the time required for full association or dissociation. It seems likely that the binding kinetics of the biosensors are dependent on the properties of the inserted riboswitch aptamer. It was previously observed that the WT Vc2-Spinach biosensor binds relatively slowly to cdiG, similar to the isolated WT Vc2 aptamer.[3,14] Thus, it seems that the yuaA aptamer has relatively slower binding kinetics than the Sc1 aptamer assayed here. Since the dynamics of cdiA regulation have not previously been studied, these two biosensors provide two different kinetic regimes for studying different reaction timescales.

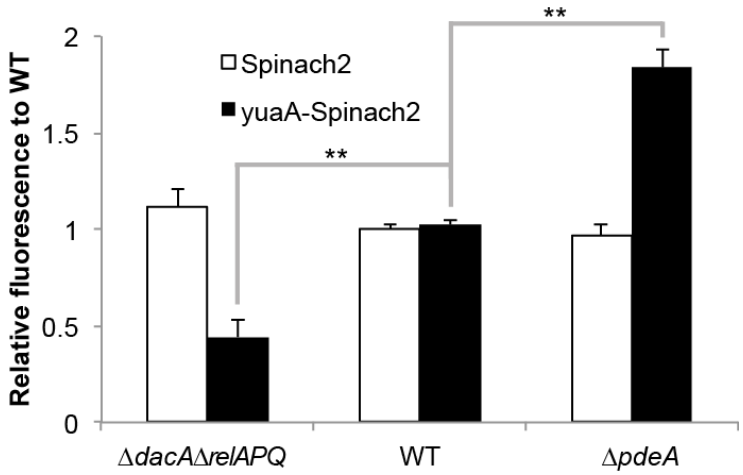



**Figure S6.** Fluorescence of Spinach2 tRNA is unchanged between tested strains of *L. monocytogenes*, whereas the yuaA-Spinach2 biosensor responds to cdiA levels. Flow cytometry analysis of *L. monocytogenes* strains expressing the Spinach2 or yuaA-Spinach2 biosensor and incubated with DFHBI-1T. Fluorescence values were normalized to the values in the WT strain (4180 ± 125 for Spinach2 and 829 ± 24 for yuaA-Spinach2). Error bars represent the standard deviation of the mean of three independent biological replicates. ** indicates $p<0.01$.

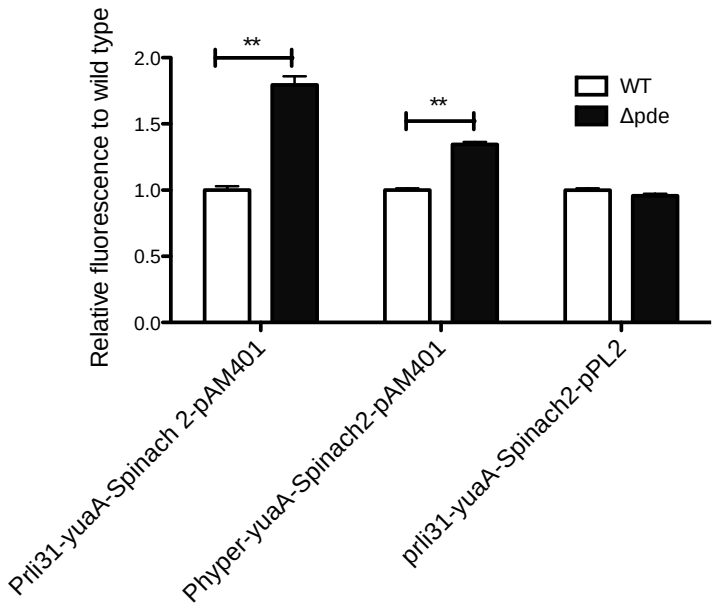



**Figure S7.** Promoter and plasmid copy number affects biosensor performance in live *L. monocytogenes*. Different promoters (Pli31 and Phyper) and shuttle plasmids (high copy vector pAM401 and integration vector pPL2) were analyzed for biosensor expression in *L. monocytogenes*. Fluorescence signal for each construct was evaluated by flow cytometry analysis in WT and Δ*pde* strains after incubation with DFHBI-1T. Fluorescence values were normalized to the values in the WT strain (610 ± 25 for Pli31 in pAM401, 434 ± 6 for Phyper in pAM401, and 246 ± 4 for Pli31 in pPL2). Error bars represent the standard deviation of the mean of three independent biological replicates. ** indicates $p<0.01$.

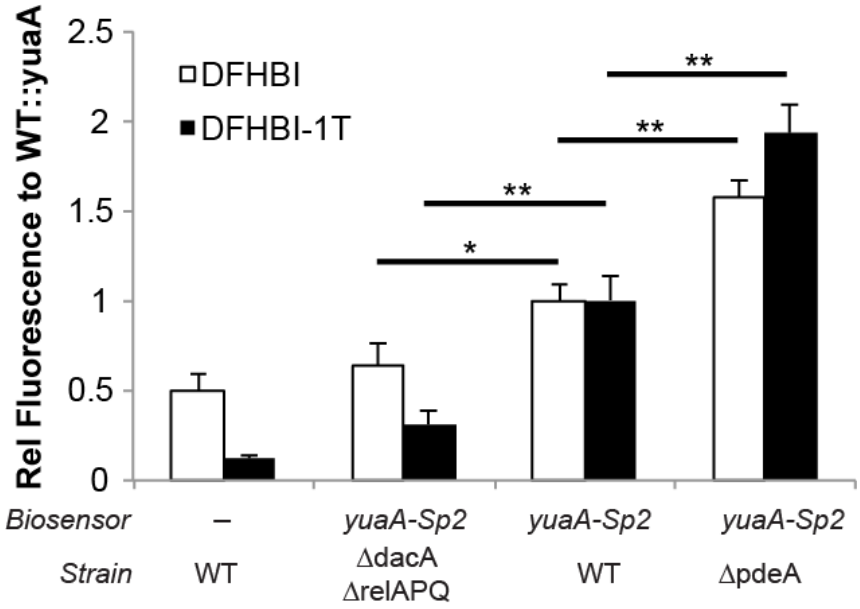



**Figure S8.** DFHBI-1T provides enhanced fluorescent signal-to-background relative to DFHBI. Data show flow cytometry analysis of *L. monocytogenes* strains incubated with either DFHBI or DFHBI-1T. Fluorescence values were normalized to the values in the WT strain harboring the biosensor (252 ± 24 for DFHBI, 735 ± 102 for DFHBI-1T). Error bars represent the standard deviation of the mean of four independent biological replicates. * indicates $p<0.05$, and ** indicates $p<0.01$.

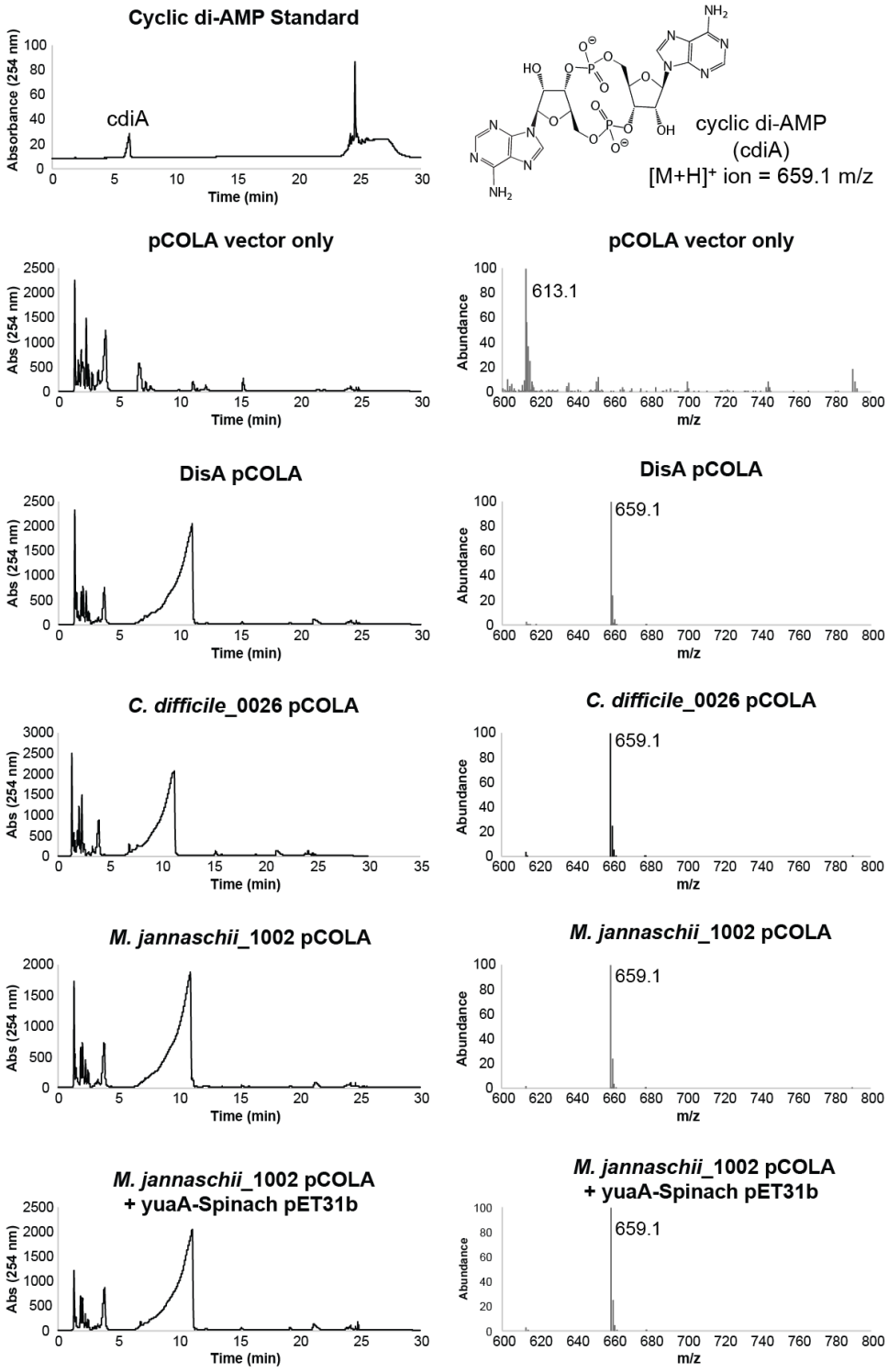



**Figure S9.** Cell extract analyses of *C. difficile* and *M. jannaschii* diadenylate cyclases. HPLC traces (left) and mass spectra for the product peak in the retention time range of the cdiA standard (right, *m/z* range = 600 to 800) are shown for cell extracts of *E. coli* expressing vector only (negative control), DisA (positive control), or putative diadenylate cyclases from *C. difficile* and *M. jannaschii*.

**Table S1.** Values for flow cytometry analysis that are graphed in Figure 3b-c.

| | No Enzyme | *P. fluorescens* WspR | *V. cholerae* DncV | *B. subtilis* DisA | *M. jannaschii* _1002 | *C. difficile* _0026 |
|---|---|---|---|---|---|---|
| **cdiG biosensor** | 568 ± 72 | 1928 ± 249 | 747 ± 176 | 928 ± 352 | 425 ± 7 | 350 ± 5 |
| **cAG biosensor** | 434 ± 2 | 372 ± 8 | 3960 ± 20 | 694 ± 27 | 661 ± 14 | 649 ± 2 |
| **cdiA biosensor** | 387 ± 8 | 381 ± 9 | 842 ± 12 | 4610 ± 30 | 2690 ± 130 | 5080 ± 910 |

Data represent the mean and standard deviation of three independent replicates from one representative experiment.

These data (also graphed in Figures 3b, c) show that each fluorescent biosensor clearly responds to co-expression of the corresponding synthases, with 3 to 12-fold increase in fluorescence. In some cases, we also see modest (< 2-fold) fluorescence increases upon co-expression of non-matched synthases. This effect may be due to several factors: (i) slight cross-sensitivity of the biosensor (the cdiG biosensor has poor affinity for the ligand cAG, and vice versa); (ii) minor differences in biosensor levels upon co-expression of different enzymes (see Fig. S8 in reference 3); (iii) in the case of the cdiG biosensor, expression of other synthases may indirectly impact endogenous cdiG metabolism in *E. coli*. This analysis demonstrates that fluorescent biosensors with high fluorescence activation are needed in order to overcome the inherent sources of variability associated with performing the experiments *in vivo*. In particular, an improved biosensor for cdiG is needed and will the subject of a future publication.

The difference in fluorescence signal for *M. jannaschii* and *C. difficile* enzymes can be due to changes in several factors: (i) biosensor levels upon co-expression of different enzymes (measured to be negligible to less than 2-fold, see Fig. S8 in reference 3; (ii) protein expression levels; (iii) enzyme activity levels. If it is desired to compare enzyme activity levels, normalization to biosensor and protein levels would be required. For in vivo experiments, the former can be accomplished by normalizing to Spinach2 fluorescence, which has to be performed as a separate control experiment. The latter can be accomplished via tagging the enzymes with a fluorescent protein such as mCherry, which gives a fluorescence signal at a different wavelength than the biosensor.

**Table S2.** Nucleotide sequences of Spinach2 constructs from Figure 1b. The Spinach2 sequence is represented in uppercase letters and ydaO and yuaA sequences are lowercase. Regions of the P1 step predicted to base pair are highlighted in yellow.

| Construct | Sequence(5'→3') |
|---|---|
| ydaO P1-7 | GATGTAACTGAATGAAATGGTGAAGGACGGGTCCAaatcgcttaatctgaaatcagagcgggggacc caatagaacggctttttgccgttggggtgaatccttttaggtagggctaactctcatatgcccgaa tccgtcagctaacctcgtaagcgttTTGTTGAGTAGAGTGTGAGCTCCGTAACTAGTTACATC |
| ydaO P1-7+PK | GATGTAACTGAATGAAATGGTGAAGGACGGGTCCAaatcgcttaatctgaaatcagagcgggggacc caatagaacggctttttgccgttggggtgaatccttttaggtagggctaactctcatatgcccgaa tccgtcagctaacctcgtaagcgttcgtgagaggaTTGTTGAGTAGAGTGTGAGCTCCGTAACTAGT TACATC |
| ydaO P1-5 | GATGTAACTGAATGAAATGGTGAAGGACGGGTCCAcgcttaatctgaaatcagagcgggggacccaa tagaacggctttttgccgttggggtgaatccttttaggtagggctaactctcatatgcccgaatcc gtcagctaacctcgtaagcgTTGTTGAGTAGAGTGTGAGCTCCGTAACTAGTTACATC |
| ydaO P1-4 | GATGTAACTGAATGAAATGGTGAAGGACGGGTCCAgcttaatctgaaatcagagcgggggacccaat agaacggctttttgccgttggggtgaatccttttaggtagggctaactctcatatgcccgaatccg tcagctaacctcgtaagcTTGTTGAGTAGAGTGTGAGCTCCGTAACTAGTTACATC |
| ydaO P3-7 | GATGTAACTGAATGAAATGGTGAAGGACGGGTCCAgcgggggacccaatagaacggctttttgccgt tggggtgaatccttttaggtagggctaactctcatatgcccgaatccgtcagctaacctcgtTTGT TGAGTAGAGTGTGAGCTCCGTAACTAGTTACATC |
| ydaO P3-5 | GATGTAACTGAATGAAATGGTGAAGGACGGGTCCAgggggacccaatagaacggctttttgccgttg gggtgaatccttttaggtagggctaactctcatatgcccgaatccgtcagctaacctcTTGTTGAG TAGAGTGTGAGCTCCGTAACTAGTTACATC |
| yuaA P1-7+PK | GATGTAACTGAATGAAATGGTGAAGGACGGGTCCAtaccgcttaatcaaacacgaacgggggaacca acgattggctgttttattaacagccttggggtgaatcttactaagtaagaggggtactctgaatcc ctaatccgacagctaacctcgtaggcgtatacagagaggaggTTGTTGAGTAGAGTGTGAGCTCCGT AACTAGTTACATC |
| yuaA P1-6 | GATGTAACTGAATGAAATGGTGAAGGACGGGTCCAaccgcttaatcaaacacgaacgggggaaccaa cgattggctgttttattaacagccttggggtgaatcttactaagtaagaggggtactctgaatccc taatccgacagctaacctcgtaggcgtTTGTTGAGTAGAGTGTGAGCTCCGTAACTAGTTACATC |
| yuaA P1-5 | GATGTAACTGAATGAAATGGTGAAGGACGGGTCCAcgcttaatcaaacacgaacgggggaaccaacg attggctgttttattaacagccttggggtgaatcttactaagtaagaggggtactctgaatcccta atccgacagctaacctcgtaggcgTTGTTGAGTAGAGTGTGAGCTCCGTAACTAGTTACATC |
| yuaA P1-4 | GATGTAACTGAATGAAATGGTGAAGGACGGGTCCAgcttaatcaaacacgaacgggggaaccaacga ttggctgttttattaacagccttggggtgaatcttactaagtaagaggggtactctgaatccctaa tccgacagctaacctcgtaggcTTGTTGAGTAGAGTGTGAGCTCCGTAACTAGTTACATC |
| yuaA P1-3 | GATGTAACTGAATGAAATGGTGAAGGACGGGTCCActtaatcaaacacgaacgggggaaccaacgat tggctgttttattaacagccttggggtgaatcttactaagtaagaggggtactctgaatccctaat ccgacagctaacctcgtaggTTGTTGAGTAGAGTGTGAGCTCCGTAACTAGTTACATC |
| yuaA P1-2 | GATGTAACTGAATGAAATGGTGAAGGACGGGTCCAttaatcaaacacgaacgggggaaccaacgatt ggctgttttattaacagccttggggtgaatcttactaagtaagaggggtactctgaatccctaatc cgacagctaacctcgtagTTGTTGAGTAGAGTGTGAGCTCCGTAACTAGTTACATC |
| yuaA P3-7 | GATGTAACTGAATGAAATGGTGAAGGACGGGTCCAacgggggaaccaacgattggctgttttattaa cagccttggggtgaatcttactaagtaagaggggtactctgaatccctaatccgacagctaacctc gtTTGTTGAGTAGAGTGTGAGCTCCGTAACTAGTTACATC |
| yuaA P3-5 | GATGTAACTGAATGAAATGGTGAAGGACGGGTCCAgggggaaccaacgattggctgttttattaaca gccttggggtgaatcttactaagtaagaggggtactctgaatccctaatccgacagctaacctcTT GTTGAGTAGAGTGTGAGCTCCGTAACTAGTTACATC |

**Table S3.** Riboswitch aptamer sequences tested in this study. All had the Spinach2 sequence appended: `5′-GATGTAACTGAATGAAATGGTGAAGGACGGGTCCA – inserted riboswitch sequence – TTGTTGAGTAGAGTGTGAGCTCCGTAACTAGTTACATC-3′`

| Name | Species | Bit score | Accession Number | Sequence (P1-4 variants) |
|---|---|---|---|---|
| **Cp1** | *Carboxydibrachium pacificum* DSM 12653 | 117.84 | ABXP01000066 | cgctgaattcgttaagaagcgggggaccc actcttttggggtgaatccatcttttaaa agatggtagggttatctttcgacccgaac ccgtcagctaaccccgtaagcg |
| **Ba1** | *Bacillus anthracis* str. A2012 | 113.47 | AAAC01000001a | cgctgagttccagaaatggagcgggggaa ccaattttgtgcatcgtcacttggggtga atctttcagttttgaaagtagggctactc tttaggcccgaatccgacagctaacctcg taagcg |
| **Ti1** | *Thermoanaerobacter italicus* Ab9 | 109.36 | CP001936a | cgctgaattcgtaaagaagcgggggaacc actttgggggtgaatccatcttttgataa gatggtagggttatctttcgacccgaacc cgtcagctaacctcgtaagcg |
| **Ba2** | *Bacillus anthracis* str. A2012 | 107.7 | AAAC01000001c | cgctgagtccaattatatggagcggagga accaatttgtgcagtgtcactcggggtga atctttcaattttgaaagtagggctactc tcaaagcccgaatccgacagctaacttcg taagcg |
| **Bc1** | *Bacillus cereus* W | 101.83 | ABCZ02000003 | cgctgaaaccgcatggtgcgggggacccg ttcttatggattagttcttaatccaatgg ggtgaatccttgaaaaaggtagggctact cataggcccgaatccgacagctaacctcg taagcg |
| **Te1** | *Thermoanaerobacter ethanolicus* JW 200 | 96.92 | AEYS01000078 | tgccgaatccgaaaggtacggaggaaccg ctttttggggttaatctgtagctttaagc tgcagtagggataccttctgtcccgcacc cgacagctaactccggaggca |
| **Cb1** | *Clostridium botulinum* A str. ATCC 3502 | 94.71 | AM412317a | gcctaagtttttttaaaacacggggaaacc aaattttggggtgaatcgtaaaattacgt aggttatacctttttccgaacccgtcagct aaccccgtaggc |
| **Bc2** | *Bacillus cereus* AH1134 | 93.71 | ABDA02000002 | ttgctgagtccaaattttggagcgggggа accatttttgtagctatgctacttggggc gaatctttttaagtagggaaactctcac ttcccgagtccgacagctaacctcgtaag cgtaa |
| **Bco1** | *Bacillus coagulans* XZL4 | 91.84 | AFWM01000138 | cgctcaattacatgtttatgtaaacgggg gaaccaacgggtgctttgggcaccaggggg tgaaacctttcattgaaaggtagggaaac tctttattccctaatccgtcagctaacct cgtaagcg |
| **Gsp1** | *Geobacillus* sp. C56-T3 | 90.42 | CP002050 | cgctgaatccggcatggagcgggggaccc gatttggcagcgtctgctgtcttggggtg aatccgaaaggacgggcatctctccgagc ccgaacccgacagctaacctcgcaagcg |
| **Cb2** | *Clostridium botulinum* D str. 1873 | 89.05 | ACSJ01000007 | tgccgaatctctattaggagtacgggggа actattttggggtgaaacttattcttcac taagtggggttcctcaaccctaacccgtc aactaacctcggaggca |

| | | | | |
|---|---|---|---|---|
| **yuaA** | *Bacillus subtilis* BSn5 | 87.71 | CP002468 | gcttaatcaaacacgaacgggggaaccaa cgattggctgttttattaacagccttggg gtgaatcttactaagtaagaggggtact ctgaatccctaatccgacagctaacctcg taggc |
| **Sc1** | *Streptomyces chartreusis* NRRL 12338 | 87.65 | AGDE01000176 | cgccgaatcccgaaagggaaccggggaac caccacttggggtgaatcgggcggatgcc gccgtggaagcacggtcggtatacgcgcg taggagaccttcctgctccgaacccgtca gctaacccggtaggcg |
| **Bp1** | *Bacillus pseudofirmus* OF4 | 87.63 | CP001878 | gcttaaactgacaaggattacacctttt agtgcgggggaaccaatgaatttggcaag gattatattgtcttggggtgagtcttcat aattgaagaagggatactctcagttccta acccgacagctaactccgtaagc |
| **Fm1** | *Fusobacterium mortiferum* ATCC 9817 | 87.36 | ACDB02000009 | gcttaatctatttttaggataaaaatataga gcgggggacccaaccttcttggggcgtat ttctcaattgagaataggatacttttcaa tccgagcccgacagctaacctcgtcagc |
| **Cp2** | *Clostridium papyrosolvens* DSM 2782 | 86.88 | ACXX02000019 | tgctgaatcttcgccaaaagcgcgaagta cggaggaaccaagaaaaaggggggttaatc cattttatatggtagggtgatcctattac ccgaacccggcagctaacttcggaagca |
| **Ti2** | *Thermoanaerobacter italicus* Ab9 | 86.45 | CP001936b | tgccgaatccccgataaggggtacggggg aattaaattggggtgaatccactttgtgg tagggtggccctcacccgaacccgtcaac taacctcggaggcg |
| **Cc2** | *Clostridium carboxidivorans* P7 | 85.38 | ADEK01000001 | gcctaagtttttattaaacacggggaaa ccggaatttttggggtgaatcgtatttt acgtaggttatgcctttaccgaacccgac agctaaccccgtaggt |
| **Dm1** | *Desulfitobacterium metallireducens* DSM 15288 | 84.56 | AGJB01000001 | ggcctaattcctaaggggaaacgggggaa ccacttttggggtgaatcgcatcttatg cgtagagcgtgcgctcgaatccgtcagct aacctcgtaagct |
| **Dm2** | *Desulfitobacterium metallireducens* DSM 15288 | 83.69 | AGJB01000014 | agctgaatctggtttttccagaacggggg atctcgtactggggtgtatcgtgccgtta ggtgcgtagggctacacctgtgcccaaac ccgtcaactaacctcgtaagct |
| **Ca1** | *Clostridium acetobutylicum* ATCC 824 | 83.59 | AE001437 | tgccgaatcttaataatatttaagtacgg gggaacacattgtgggtgaatcttacgac tattcgtaagtagggtgccttcaacccga atccgtaagctaacctcggaggca |
| **Ac1** | *Acidothermus cellulolyticus* 11B | 81.03 | CP000481 | cgccgaacctgcctcgcaggaccggggac ccagcaccggcgcacagcgcccaggggtg aatccggtgttcgcaccggtagggcgatc ttccccgcccgaacccgtcagctaacccg gtaggcg |
| **Cs1** | *Clostridium sporogenes* ATCC 15579 | 77.48 | ABKW02000004 | tgccgaatcttctttatgaagtaccgggg atatctttggggtgaatctcttaggaata gggtacctctaacccgaacccgtcaacta acctgggaggca |
| **Gs1** | *Geobacter sulfurreducens* PCA | 66.24 | AE017180 | gccgagatccgttcgcgagaacgggtgcg ggggacccacttcgggggcgtattccggc aatggcgccggatagaggactttcagcct cgagcccgtcagctaacctcgtaggc |

**Table S4**. List of promoter and synthetic DNA for *L. monocytogenes* and *E. coli* studies

| Name | Sequences |
|---|---|
| Rli31 promoter | GCCAATTCCTCCTATATATAAGATATTTCCTAGTTAATTATAGCATAGT<br>AAAGAAAAGATAGAAAAATATCACGATTATTGTATTAATGTTACATTTT<br>ATTATTAAAACAGCATTTTTGATTTTTATGAAATATTTTTATAATAAAA<br>GTATTGACTAAGACATAAAAGTAATGTAAGATATACTTGCCCGGATAGC<br>TCAGTCGGTAGAGCAGCGGCCG |
| Phyper promoter | AATTTTGCAAAAAGTTGTTGACTTTATCTACAAGGTGTGGCATAATGTG<br>CGTCGGCCGCTCTAGAACTAGTGGATCC |
| *Clostridium difficile disA*-like gene codon optimized | ATGGAAAACTTTCTCGACAACAAAAATATGCTTTATGCGTTGAAAATGA<br>TCTCTCCGGGGACACCGCTGCGTTTAGGTCTGAATAACGTTTTGCGGGC<br>GAAAACGGGCGGCTTAATCGTAATTGCTACGAACGAAGATGTTATGAAA<br>ATCGTCGATGGGGGGTTCGCGATCAACGCGGAATATAGCCCGTCTTATC<br>TGTATGAATTGGCCAAAATGGATGGTGCTATCGTCTTGTCTGGTGACGT<br>TAAAAAAATTCTGTTTGCGAACGCGCAACTTATTCCCGACTATTTCATC<br>GAAACGAGCGAAACCGGCACGCGTCATCGGACAGCAGAGCGTGTGGCCA<br>AACAAACCGGAGCCATCGTAATTGGCATCTCACAGCGTCGTAATGTTAT<br>CACGGTGTACCGCGGCAATGAAAAATATGTCGTCGAAGACATCAGCAAA<br>ATCTTTACTAAGGCAAACCAGGCTATCCAAACCTTGGAGAAATACAAAA<br>CCGTTCTTGATCAGGCCGTGACAAATCTGAATGCCCTGGAATTCAATGA<br>CCTGGTTACCATCTATGATGTCGCACTGGTCATGCAGAAAATGGAGATG<br>GTTATGCGGGTAACCAGCATTATCGAAAAATACGTGATCGAACTGGGCG<br>ATGAGGGTACGCTGGTATCGATTAACTAG |
| *Methanocaldococcus jannaschii disA*-like gene codon optimized | ATGATCGCTAAGTATATTATTAAACATGGGCTTGAATTGGCTTACGATA<br>TTAAAGCAGATGCATTTATGATCTTTACCGAAACAGGGAAGTCGTATGA<br>ACTTTTGAAGTCTTTCCTGAAAAAAGACGAGCACAGCGGTATTATCAAA<br>ATTTTGGACAAAATTTCTCATAAAAACGTGAAAATTATTGTGGCGACCC<br>CAAATCAGGTTACCTATAAAAAAATCTCCAGTGAGAATGAAGAAAATAT<br>TTATCCCATTTTCATTAAACATCGCGAAGATAACCGCTGCATGATTATT<br>AGTAGTGGAATTGTGCATGCCCTGAAAATGAAAATTCTGAAGGAAAATA<br>ATAAGATTGTGGCGGTTGTCGGCGAGCCCAAGACCCCGGGCAAACTGGA<br>CACCATCATGGTAGTAAATGTAAAAGAACACGTGAAGACTATTACACTG<br>TATGAACTGTTCGAGACGCTGGATGAAAAACAGAAACGTACTCTGAAAG<br>AGATTATTAAACTGGCAATGGAAATTGGTCGGGAAGGCCGTGAAGGCGA<br>ATATGTGGGCACAATCTTCGTAATGGGTGATACGCTGAATGTGATGTCA<br>ATGAGCAAACCGTTGATTTTAAATCCATTTGCCGGGCACAATGCAAGCA<br>TTTTTGACGAGAACGTGAAAGGGACCATCAAAGAACTGAGCAGCATCGA<br>TGGAGCCTTCATCATTACGGATGAAGGTAAAGTTGTCTCGGCTGGACGT<br>TTTCTCGAGATCAAGGGCGATGTCAACATCCCGAAGGGATTAGGCGCGC<br>GCCATCTTGCAGCGGCGAGCATTAGCAAAAACACGAACGCCATTGCAGT<br>TACCGTGTCTCAGTCAGGTGGTATCGTGCGCGTCTTTAAAGATGGCAAA<br>ATTGTTTTTGAAACGGATCCGCGCGCGAATATTCTCTTTTTCGACTAG |

**Table S5.** List of *L. monocytogenes* strains and plasmids used in this study.

| Strains | Source or reference |
|---|---|
| *10403s* | Bishop, D.K. and Hinrichs, D.J. (1987)[15] |
| *10403s Δpde (Lmo 0052)* | Witte, C.E. et al. (2013)[16] |
| *10403s tetR (Lmo 2589)::Tn917* | Crimmins, G.T. et al. (2008)[17] |
| *10403s ΔmdrM (Lmo1617)* | Crimmins, G.T. et al. (2008)[17] |
| *10403s ΔdacAΔrelAPQ* | Whiteley, A.T. et. al. (2015)[18] |
| *10403s::Pli31-yuaA-spinach2-pAM401* | This study |
| *10403s Δpde::Pli31-yuaA-spinach2-pAM401* | This study |
| *10403s ΔmdrM::Pli31-yuaA-spinach2-pAM401* | This study |
| *10403sΔdacAΔrelAPQ::Pli31-yuaA-spinach2-pAM401* | This study |
| *10403s tetR::Tn917::Pli31-yuaA-spinach2-pAM401* | This study |
| *10403s::Pli31-spinach2-pAM401* | This study |
| *10403s Δpde::Pli31-spinach2-pAM401* | This study |
| *10403s ΔmdrM::Pli31-spinach2-pAM401* | This study |
| *10403sΔdacAΔrelAPQ::Pli31-spinach2-pAM401* | This study |
| *10403s tetR::Tn917::Pli31-spinach2-pAM401* | This study |
| *10403s::Phyper-yuaA-spinach2-pAM401* | This study |
| *10403s Δpde:: Phyper -yuaA-spinach2-pAM401* | This study |
| *10403s ΔmdrM:: Phyper -yuaA-spinach2-pAM401* | This study |
| *10403sΔdacAΔrelAPQ:: Phyper -yuaA-spinach2-pAM401* | This study |
| *10403s::Pli31-yuaA-spinach2-pAM401* | This study |
| *10403s Δpde::Pli31-yuaA-spinach2-pPL2* | This study |
| *10403s ΔmdrM::Pli31-yuaA-spinach2-pPL2* | This study |
| *10403sΔdacAΔrelAPQ::Pli31-yuaA-spinach2-pPL2* | This study |
| *10403s tetR::Tn917::Pli31-yuaA-spinach2-pPL2* | This study |
| *10403s::Pli31-spinach2-pPL2* | This study |
| *10403s Δpde::Pli31-yuaA-spinach2-pAM401+pde-pPL2e* | This study |
| *10403s Δpde:: Pli31-yuaA-spinach2-pAM401+pdeΔPAS(74-137)-pPL2e* | This study |
| *10403s Δpde:: Pli31-yuaA-spinach2-pAM401+pdeΔGGDEF(138-301)-pPL2e* | This study |
| *10403s Δpde:: Pli31-yuaA-spinach2-pAM401+pdeΔDHH(335-449)-pPL2e* | This study |
| *10403s Δpde:: Pli31-yuaA-spinach2-pAM401+pdeΔDHH1(588-647)-pPL2e* | This study |

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