Cyclic dinucleotides are an expanding class of signaling molecules that control many aspects of bacterial physiology. A synthase for cyclic AMP-GMP (cAG, also referred to as 3′-5′, 3′-5′ cGAMP) called DncV is associated with hyperinfectivity of Vibrio cholerae but has not been found in many bacteria, raising questions about the prevalence and function of cAG signaling. We have discovered that the environmental bacterium Geobacter sulfurreducens produces cAG and uses a subset of GEMM-I class riboswitches (GEMM-Ib, Genes for the Environment, Membranes, and Motility) as specific receptors for cAG. GEMM-Ib riboswitches regulate genes associated with extracellular electron transfer; thus cAG signaling may control aspects of bacterial electrophysiology. These findings expand the role of cAG beyond organisms that harbor DncV and beyond pathogenesis to microbial geochemistry, which is important to environmental remediation and microbial fuel cell development. Finally, we have developed an RNA-based fluorescent biosensor for live-cell imaging of cAG. This selective, genetically encodable biosensor will be useful to probe the biochemistry and cell biology of cAG signaling in diverse bacteria.

cGAMP | cyclic dinucleotides | fluorescent biosensor | bacterial signaling | gene regulation

Cyclic dinucleotides are a structurally related class of second messengers that are produced as key intracellular signals triggering physiological changes in response to environmental stimuli (SI Appendix, Fig. S1). Cyclic di-GMP (cdiG) was first identified to be widespread in bacteria through the prevalence of GGDEF domain-containing proteins encoded in sequenced genomes and studied for its regulation of the critical transition from motile to sessile, biofilm-forming lifestyles (1, 2). Recently, two other cyclic dinucleotides, cyclic di-AMP (cdiA) and cAMP-GMP (cAG or 3′-5′ cGAMP; Fig. 1), have been discovered in bacteria (7, 8). Furthermore, an isomer of cAG containing a noncanonical 2′-5′ phosphodiester linkage (2′,3′-cGAMP) has been found to be produced in response to cytosolic DNA in mammalian cells (9–12). The mammalian sensor STING is stimulated by both bacterial cyclic dinucleotides and 2′,3′-cGAMP to activate the innate immune response (13). In bacteria, however, specific recognition of each second messenger is necessary to deconvolute the signaling pathways.

Of the three bacterial cyclic dinucleotides, the least is known about the signal transduction pathways for cAG (Fig. 1A). So far, the only component that has been identified is the Vibrio cholerae cAG synthase, DncV, which has homology to the mammalian cGAS enzyme through its 2′,5′-oligoadenylate synthase (OAS1) domain (8), but few homologs are found encoded in other bacterial genomes. To our knowledge, no sensors or effectors that respond selectively to cAG have yet been reported, although the second messenger has been shown to affect chemotaxis and intestinal colonization of V. cholerae (8). In addition, it remains an open question how prevalent cAG signaling is in bacteria.

Previously, we developed an RNA-based fluorescent biosensor that could be used as a tool to visualize cdIG signaling in vivo (14). This biosensor was created by fusing a natural cdIG-sensing GEMM-I class riboswitch aptamer (Vc2) (15) to the Spinach aptamer (Fig. 1B and SI Appendix, Fig. S1 B and C) (16, 17). Although the WT Vc2 aptamer is highly selective for cdIG (15), we showed that engineering the binding pocket through a G20A mutation altered ligand selectivity to render the aptamer capable of binding cAG and cdIG with similar affinities (14). Intriguingly, prior phylogenetic analysis revealed that, whereas 76% of the sequences in the GEMM-I riboswitch class have a G at this position (referenced hereafter as G20, following the Vc2 numbering) (18) shown in crystal structures of the riboswitch-ligand complex to form a Hoogsteen base pair with cdIG (3, 4), 23% instead had an A20 that presumably allows for recognition of cAG. We thus surveyed several of these A20 phylogenetic variants through the fluorescent biosensor screen to determine if any GEMM-I sequences are naturally selective for cAG.

A screen of natural A20 GEMM-I sequences revealed that the majority display promiscuous binding to cAG and cdIG, similar to G20A Vc2-Spinach (SI Appendix, Fig. S2A). A few sequences retain cdIG selectivity or have undetermined selectivities. However, one initial candidate, Gm0970, demonstrated a marked selectivity for cAG (Fig. 1C and SI Appendix, Fig. S2B). This sequence is from the bacterium Geobacter metallireducens GS-15, which has 17 predicted GEMM-I riboswitches annotated in its genome (GenBank accession NC_007517.1). Analysis of all GEMM-I sequences from this species revealed that 15/17

Significance

Bacteria are hidden forces of nature. For example, Geobacter bacteria play important roles in geochemistry by reducing metals in the environment. Scientists also are exploring the application of these bacteria toward toxic metal remediation and as “living batteries” that can generate electricity from biowaste. However, there is limited understanding of the signaling pathways that regulate this extracellular metal-reducing activity. Here we have discovered that Geobacter sulfurreducens use riboswitch sensors for a signaling molecule called cAG to regulate this process, which is an unexpected finding because cAG was previously associated only with pathogenic bacteria. Furthermore, we have adapted the riboswitch to generate a fluorescent biosensor that can be used to visualize cAG signaling in live bacteria.


The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

Data deposition: The sequences reported in this paper are provided in SI Appendix. For a list of relevant GenBank accession numbers, see SI Appendix, Tables S2 and S3.

1C.A.K. and S.C.W. contributed equally to this work.

2To whom correspondence should be addressed. Email: mingch@berkeley.edu.

This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1419328112/-/DCSupplemental.
Fig. 1. GEMM-I riboswitches from *Geobacter* bacteria are selective for cAG. (A) Cyclic AMP-GMP signaling. (B) Sequence and secondary structure model of GEMM-I-Spinach fusion constructs based upon the crystal structures of the individual RNA components (3–6). The consensus for cAG-selective riboswitches in the GEMM-Ib subclass is shown. (C) Spinach-based selectivity screen of GEMM-I riboswitch aptamers from *G. metallireducens*. The initial candidate from the A20 screen is underlined (SI Appendix, Fig. S2). All sequences have an A20 except for those marked with an asterisk, which have a G20. (D) In-line probing analysis of cyclic dinucleotide selectivity for the Gm0970 riboswitch aptamer (also see SI Appendix, Fig. S4). No reaction (NR), treatment with RNase T1 (T1), which cleaves after G nucleotides, and partial base hydrolysis (OH) serve as control and ladders. Blue arrowheads indicate nucleotides that undergo conformational change in response to ligand binding.

contain an A20 and are selective for cAG or have undetermined selectivities (Fig. 1C and SI Appendix, Table S1). The remaining 2/17 contain a G20, and one binds both cAG and cdig, whereas the other has undetermined selectivity. A similar screen of all GEMM-I sequences in *Geobacter sulfurreducens* *Geobacter* sulfurreducens strain PCA and select sequences in other *Geobacter* confirmed that the majority of GEMM-I sequences from *Geobacter* species preferentially bind cAG (SI Appendix, Fig. S3).

To validate that these riboswitch aptamers are naturally selective for cAG and that selectivity is not altered through the context of the Spinach aptamer, we performed an in-line probing analysis on the isolated Gm0970 and Gs1761 aptamers (Fig. 1D and SI Appendix, Figs. S4 and S5). Ligand-induced changes in the RNA fold are revealed by alteration of the in-line probing pattern, which corresponds to the extent of RNA self-cleavage at each nucleotide position. The Gm0970 aptamer has a dissociation constant ($K_d$) of ~16 nM for cAG and ~26 μM for cdig, which means it is ~1,600-fold selective for cAG over cdig. Similarly, the Gs1761 aptamer has a $K_d$ of ~530 pM for cAG and ~660 nM for cdig and thus is ~1,200-fold selective. Furthermore, high concentrations of up to 0.5 mM of cdig or 2.3 μM cGAMP induced very little conformational change in either aptamer, indicating that the two riboswitch aptamers do not recognize these other cyclic dinucleotides. These data also demonstrate that natural GEMM-I riboswitches from *Geobacter* bind cAG selectively. To our knowledge, these RNAs are the first specific effectors for cAG that have been identified. These sequences constitute a previously unidentified subclass of GEMM-I riboswitches, herein termed GEMM-Ib, that respond selectively to cAG (Fig. 1B).

The cyclic dinucleotide content of *Geobacter* bacteria has not been analyzed previously, although bioinformatics analyses have predicted the presence of enzymes related to cdig and cdIA signaling (19, 20) and corresponding GEMM-I and ydaO class riboswitches (15, 21). Our current results strongly suggest that these organisms also produce cAG as an endogenous signaling molecule, even though their genomes do not harbor any homologs of DncV. Thus, we performed organic-aqueous extraction of cyclic dinucleotides from *G. sulfurreducens* PCA cells. Liquid chromatography–mass spectrometry (LC-MS), MS/MS, and high-resolution mass spectrometry (HRMS) analysis revealed that all three bacterial cyclic dinucleotides are present in *G. sulfurreducens* cell extracts (Fig. 2 and SI Appendix, Figs. S6–S8). Only cdig is observed when cells are grown to a similar density in medium without supplemented yeast extract, and the corresponding analysis of 100% yeast extract did not yield any signal for cyclic dinucleotides (SI Appendix, Figs. S9 and S10). We detected a mass consistent with cAG in a miniscule amount only after analyzing concentrated HPLC fractions collected in the retention time range for cyclic dinucleotides (SI Appendix, Fig. S10). Thus, the amount of cAG isolated from the *G. sulfurreducens* cell extracts far exceeds the total amount that can be scavenged from the media, which implies that de novo synthesis of cAG is occurring. These experiments do not rule out a role for import mechanisms, but nevertheless demonstrate the presence of cAG as a native signaling molecule in *G. sulfurreducens*. Based on the ligand selectivity of their riboswitches, we expect cAG to be present in other *Geobacter* species as well. These results are significant because they provide, to our knowledge, the first evidence that cAG signaling extends to delta proteobacteria and, furthermore, beyond bacteria that harbor homologs of DncV.

We predict that the signaling molecule cAG turns on the expression of genes or operons associated with GEMM-Ib riboswitches via regulating the formation of rho-independent transcription terminators (SI Appendix, Tables S1 and S2). For example, the Gs1761 riboswitch upstream of the pgcA gene is expected to form a terminator stem with portions of the aptamer domain, but the terminator would be disrupted in the cAG-bound structure, as the P1 and P3 stem act as antiterminator sequences (Fig. 3A). In-line probing analysis of an extended riboswitch sequence in the presence of cAG reveals pattern changes consistent with both P1 and P3 stem formation and with disruption of the terminator stem (Fig. 3B). These structural changes are observed at low concentrations of cAG (starting at 10 nM), but are not observed with cdig until concentrations are ~1,000-fold or greater (10–50 μM), which agrees with our previous results on selectivity for ligand binding. Thus, we demonstrate that cAG binding to the Gs1761 riboswitch aptamer disrupts terminator stem formation. Consistent with these results, an increase in full-length transcription with cAG was observed for another GEMM-Ib riboswitch (22).
A key metabolic feature of the Geobacter genus is the ability to perform extracellular electron transfer. In addition to playing an important role in natural geochemistry through the reduction of environmental metals (23), Geobacter have been described as “living batteries” that have the capacity to generate electricity from organic waste (24). Significantly, the genes regulated by GEMM-Ib class riboswitches and thus activated by cAG signaling include many that are functionally linked to this process (SI Appendix, Table S1). For example, the gene regulated by the Gs1761 riboswitch, pgcA, encodes a periplasmic cytochrome c protein that is conserved in Geobacter species and is more highly expressed during growth on insoluble Fe(III) oxide versus Fe(III) citrate (25, 26).

Interestingly, a C-to-T transition that maps to the Gs1761 riboswitch in the pgcA transcript was shown to double the rate of Fe(III) oxide reduction by G. sulfurreducens (27). We analyzed the extended C78U Gs1761 riboswitch sequence by in-line probing and found that the point mutation indeed destabilizes the terminator stem, as the observed pattern is similar to that of WT Gs1761 bound to cAG (Fig. 3B). However, unlike the WT riboswitch, the C78U mutant is insensitive to the presence of cAG or cdIG (SI Appendix, Fig. S11). This latter observation can be rationalized using the crystal structures of Vc2 bound to cdIG, which showed that the corresponding nucleotide forms a Watson-Crick base pair to the ligand (3, 4). Together, these results suggest that the C78U mutation to the riboswitch would constitutively turn on gene expression, which corroborates the ∼15-fold increase of pgcA transcripts observed in the mutant strain (27). Taken together, our data provide functional evidence for cAG regulation via a GEMM-Ib riboswitch in vitro and explain the results of a prior study that implicated riboswitch-based regulation of the pgcA gene in vivo.

Several other cytochrome c-containing and biogenesis proteins appear to be regulated by cAG, including OmcC, which has been shown to be essential for metal oxide reduction and more highly expressed upon growth on insoluble Fe(III) or Mn(IV) oxides (28, 29). Although cytochrome genes are remarkably abundant in Geobacter species, they are not well conserved, making it difficult to base functional assignments of electron conduits on phylogenetic arguments (30). The identification of a subset of cytochromes coregulated by cAG suggests that they are part of a specific pathway and distinguishes them from others encoded in the genome.

In addition to extracellular cytochromes, Geobacter produce specialized type IV pili that are intimately involved in contacting insoluble metal sources, including electrodes, and there is evidence for direct electron shuttling by the pili (31), although this mechanism is still under active debate (32). The Gm0970 and Gs2033 riboswitches reside within an operon conserved in Geobacter that contains genes encoding minor pilins and pilus assembly proteins (SI Appendix, Fig. S12). Thus, the genes of known function associated with GEMM-Ib appear to be involved in extracellular electron transfer and allow us to propose a role for cAG signaling in the regulation of Geobacter electrophysiology (Fig. 3C). More significantly, the majority of genes associated with GEMM-Ib are of unknown or uncharacterized function, including two (Gsu2033 and Gsu2885) that are cotranscribed with PilMNOPO and OmcAHG, respectively (SI Appendix, Fig. S12). An intriguing hypothesis is that these genes play a previously unrecognized role in extracellular electron transfer.

To further elucidate and study cAG-controlled pathways in Geobacter and other bacteria, we envisioned developing a genetically encodable fluorescent biosensor for live-cell imaging of cAG. The original Gm0970-Spinach construct, which harbors a 3-nt base-paired transducer stem (P1-3), displays modest (approximately twofold) fluorescence activation (Fig. 4A). Through optimization studies of the transducer stem that involved screening stems of different lengths (2–6 bp) and bulge deletions, two selective biosensors were developed that display much higher fluorescence activation (14.1-fold for P1–4 and 7.8-fold for P1–4delA) in response to cAG (Fig. 4A and Fig. S13). Both biosensors exhibit similar apparent binding affinities in the low micromolar regime and binding kinetics for cAG at 3 mM MgCl2 and 37 °C (SI Appendix, Figs. S14 and S15). Importantly, fluorescence microscopy and flow cytometry experiments demonstrate that biosensors based on the Gm0970 riboswitch aptamer function in vivo (Fig. 4B and C and SI Appendix, Figs. S16 and S17). Furthermore, these new fluorescent biosensors are able to clearly distinguish between enzymatic production of cAG versus cdIG.

It is clear that much remains to be discovered about cAG signaling. For example, whereas the cAG synthase DncV is found in V. cholerae, so far no phosphodiesterases or effectors for cAG have been identified in any organism. We now have revealed that cAG is produced by G. sulfurreducens and that Geobacter species harbor riboswitches as effectors that are selective for cAG. However, homology searches to DncV did not
GEMM-I riboswitches that we tested were selective for coexpressing P1 activation and deactivation to SI Appendix sequences harboring the A20 variation demonstrating that most bind both cyclic dinucleotides (cAG, cdiG; a few were conspicuously promiscuous for cAG and cdiG) (Fig. 1C). These experiments are ongoing in our laboratory, and we expect will provide further insight into how widespread cAG signaling is in the eubacterial kingdom.

Another noteworthy aspect of cAG as a second messenger is its structural similarity to cdiG and cdiA. Although most Geo-bacter GEMM-I riboswitches that we tested were selective for cAG, a few were conspicuously promiscuous for cAG and cdiG (Fig. 1C and SI Appendix, Fig. S3). Furthermore, a limited survey of non-Geo-bacter sequences harboring the A20 variation demonstrated that most bind both cyclic dinucleotides (SI Appendix, Fig. S24). Findings from this study lead to further intriguing questions regarding cyclic dinucleotide biology, including what is the molecular basis of selectivity versus promiscuity for different receptors and whether the hybrid nature of cAG promotes cross-receptor signaling.

Materials and Methods

General Reagents and Oligonucleotides. GEMM-I–Spinach DNA oligonucleotides were purchased as ultrasmall from Integrated DNA Technologies, and other oligonucleotides were purchased from ElBio Biopharmaceuticals. Cyclic dinucleotides were purchased from Axxora. 5′-Difluoro-4-hydroxybenzylidene imidazolinone (DFHBI) was either purchased from Lucerna or was synthesized (33) for characterization experiments. RNAs purified by PAGE were subsequently eluted from gel pieces in Crush Soak buffer (10 mM Tris HCl, pH 7.5, 200 mM NaCl, and 1 mM EDTA, pH 8.0) precipitated with ethanol, and resuspended in 1× TE buffer (10 mM Tris HCl, pH 8.0, 1 mM EDTA). RNAs purified by a 96-well spin column were purified according to the manufacturer’s protocol and were resuspended in 0.5× TE buffer. Accurate measurement of RNA concentration was determined after performing a hydrolysis assay to eliminate the hypochromic effect due to secondary structure in these RNAs (37).

In Vitro Transcription. Preparation of RNAs was carried out as previously described (14). Briefly, DNA templates for in vitro transcription were prepared via a PCR that added the T7 polymerase promoter sequence (SI Appendix, Tables S3 and S4). Templates were then transcribed using T7 RNA polymerase and were purified either by a 96-well spin column (2R 96 Clean & Concentrator, Zymo Research) for screening experiments or by denaturing (7.5 M urea) 6% (wt/vol) PAGE for characterization experiments. RNAs purified by PAGE were subsequently eluted from gel pieces in Crush Soak buffer (10 mM Tris HCl, pH 7.5, 200 mM NaCl, and 1 mM EDTA) precipitated with ethanol, and resuspended in 1× TE buffer (10 mM Tris HCl, pH 8.0, 1 mM EDTA). RNAs purified by a 96-well spin column were purified according to the manufacturer’s protocol and were resuspended in 0.5× TE buffer. Accurate measurement of RNA concentration was determined after performing a hydrolysis assay to eliminate the hypochromic effect due to secondary structure in these RNAs (37).

In Vitro Fluorescence Assays of GEMM-I–Spinach Variants. To screen GEMM-I riboswitch aptamer selectivity, a solution of ligand (0, 1, 50, or 100 μM) and DFHBI (33 μM) was prepared in buffer containing 40 mM Hepes, 125 mM KCl, and 10 mM MgCl₂ at pH 7.5. Each RNA was renatured in buffer at 70 °C for 3 min and cooled to ambient temperature for 5 min before addition to the reaction solution at a final concentration of 100 nM. Binding reactions were incubated at 30 °C in a Corning Costar 3915 96-well black plate until equilibrium was reached (see activation curves in SI Appendix, Fig. S15). The fluorescence emission was measured using a Molecular Devices SpectraMax Paradigm plate reader with the following instrument parameters: 460 nm excitation and 500 nm emission. The background fluorescence of the buffer alone (without DFHBI) was subtracted from each sample to determine the relative fluorescence units.

To analyze the selectivity or binding affinity of RNA biosensor constructs, assays were run as described above, except at 37 °C with 3 mM MgCl₂ to mimic physiological conditions. Selectivity experiments were performed using 100 nM RNA and 100 μM ligand. Experiments to measure Kₐ were performed with 30 nM RNA and various ligand concentrations, and the fluorescence of the sample with DFHBI and RNA in buffer without ligand was subtracted to determine relative fluorescence units.

Biosensor Activation and Deactivation Assays. Activation and deactivation assays were performed as previously described (14). Briefly, to determine activation rate, the ligand (30 μM) and DFHBI (33 μM) in buffer were prepared and prewarmed to 37 °C. RNA (100 nM final concentration) was renatured in buffer at 70 °C for 3 min and then cooled to 37 °C before addition to the reaction solution. Immediately after the RNA was added, the fluorescence emission was measured every 2 min at 37 °C using the same plate-reader settings as for the ligand binding assays.

Deactivation assays were performed using buffer exchange to remove the cyclic dinucleotide ligand. RNA (100 nM) was mixed with ligand (30 μM) and DFHBI (33 μM) at 37 °C in a buffer containing 40 mM Hepes, 125 mM KCl, and 3 mM MgCl₂ at pH 7.5. Once the reaction had reached maximum fluorescence, half of the reaction was passed through an Illustra MicroSpin G-25 Column (GE Healthcare) that had been equilibrated with DFHBI (33 μM).
in-line buffer: 50 mM Tris was used, m/ PNAS Early Edition
PCA and Cyclic dinucleotides
600
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ULB (urea loading buffer, half of the standard = 200 μL reactions and quenched with 90 μL ddH2O and 50 μL 2x ULB before 25 L of the sample was loaded per lane onto a 10% urea–
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In-line Probing Assays. In vitro transcribed RNA was radiolabeled with γ-32p ATP using T4 polynucleotide kinase (New England Biolabs) following standard procedures (38). After PAGE purification, RNAs were passed through an inline spin column G-25 Column (GE Healthcare) and eluted with ddH2O. In-line probing assays (1x in-line buffer: 50 mM Tris-HCl, pH 8.3, 20 mM MgCl2, 100 mM KCl) were performed as previously described (37) for the extended Gs1761 riboswitch with terminator construct. The following modifications were made to the procedure to measure ligand-binding affinities below 1 nM for the Gm0970 and Gs1761 riboswitch aptamers. The reactions were quenched with 50 μL of 2x ULB (urea loading buffer, half of the standard volume, and 25 μL of the sample was loaded per lane onto a 10% urea–PAGE gel. To achieve –2,000–4,000 cpm per lane, the RNA had to be very endonucleolytic in fumarate–acetate medium with or without yeast extract, at 30 °C until an OD260 of ~0.4 was reached, which took about 2–3 d. Cells were harvested in 50 or 100 mL aliquots by centrifugation at 10,000 × g for 10 min at 4 °C, and pellets were stored at –80 °C. The fumarate–acetate cell extracts was prepared as 10 μL reactions and quenched with 90 μL ddH2O and 50 μL 2x ULB before 25 L of the sample was loaded per lane onto a 10% urea–

Liquid Culture Growth of G. sulfurreducens PCA. From a glycerol stock, a 10 mL starter culture of G. sulfurreducens PCA in fumarate–acetate media alone or supplemented with 0.1% yeast extract was grown anaerobically at 30 °C for 5–7 d without shaking in anaerobic culture tubes sealed under nitrogen with 20 mm blue butyl rubber stoppers and aluminum crimps. Intermediate 50–50 mL cultures were inoculated at 1:10 dilution and then used to inoculate master 1 L cultures at 1:20 dilution in the same media. Cells were grown anaerobically in fumarate–acetate medium with or without yeast extract at 30 °C until an OD600 of ~0.4 was reached, which took about 2–3 d. Cells were harvested in 50 or 100 mL aliquots by centrifugation at 10,000 × g for 10 min at 4 °C, and pellets were stored at –80 °C. The fumarate–acetate media contained the following in 1 L: 4 g sodium fumarate dibasic (25 mM sodium fumarate final concentration), 1.64 g sodium acetate (20 mM final concentration), 0.49 g NaH2PO4·2H2O, 0.97 g NaHCO3, 0.25 g NH4Cl, 0.1 g KCl, 10 mL 100 μM vitamin mix (39), 1 mL 1,000× NB minerals mix (39), and 1.0 g yeast extract. Bacto brand, if used). The media was degassed and bledged by bubbling under nitrogen gas for 30 min while cooling, then aliquoted, sealed under anaerobic conditions, and autoclaved.

Cell Extraction from G. sulfurreducens PCA and E. coli. Cyclic dinucleotides were extracted as described previously (40) with the following modifications. A frozen cell pellet from 100 mL of liquid culture was thawed and resuspended in 200 μL of LiBr/oxan. Cells were grown anaerobically to an OD600 of ~0.5 and then induced with 1 mM isopropyl β-D-thiogalactopyranoside (IPTG) at 37 °C for 2 h. Cells were harvested by centrifugation at 10,000 × g for 10 min at 4 °C, and pellets were stored at –80 °C.

Direct LC-MS Analysis of Cell Extracts. LC-MS analysis of G. sulfurreducens PCA cell extracts was performed using an Agilent 6120 Quadrupole LC-MS with an Agilent 1260 Infinity liquid chromatograph equipped with a diode array detector. Sample volumes of 20 μL were separated on a Poroshell 120 EC C18 column (50 mm length × 4.6 mm internal diameter, 2.7-μm particle size; Agilent) at a flow rate of 0.4 mL/min using a linear elution program of 0–10% solvent B over 20 min, where solvent A was H2O + 0.05% TFA and solvent B was MeCN + 0.05% TFA. Under these conditions, the cyclic dinucleotides in extracts were found to elute always in the order of cdG (7.3 ± 0.3 min), cAG (7.6 ± 0.3 min), and cdA (7.9 ± 0.4 min), although there was variability in the retention times between runs. Thus, assignment of cyclic dinucleotide identity was made through analysis of the mass spectra.

Extract samples were analyzed by MS in the positive ion mode using the range of m/z = 600–800. When a broader range of 100–1,000 m/z was used, the expected mass for the corresponding cyclic dinucleotide was not fully resolved from other small molecules present in the extract. Thus, the UV absorbance peaks detected at 254 nm may not be solely attributable to the cyclic dinucleotides.

HPLC Fractionation and MS Analysis (HRMS, MS/MS) of Cell Extracts. Discrete fractions from G. sulfurreducens PCA cell extracts were collected using an Agilent 1260 Infinity liquid chromatograph equipped with a diode array detector and analytical-scale fraction collector. Extract samples of 100–150 μL were separated on a Polaris C18-A column (250 mm length × 4.6 mm inner diameter, 5-μm particle size, 180-Å pore size; Agilent) at 50 °C with a linear gradient of 0–100% solvent B over 1 min. Solvent A was 100 mM ammonium acetate, and solvent B was methanol. The elution program consisted of 0% B for 10 min, a linear gradient to 10% B over 1 min, isocratic conditions at 10% B for 4 min, a linear gradient to 30% B over 4 min, a linear gradient to 0% B over 1 min, and isocratic conditions at 0% B for 10 min. Eluted fractions were lyophilized multiple times to remove excess salts before mass spectrometry analysis.

HRMS and tandem mass spectrometry (MS/MS) measurements of collected fractions were performed using a Q-Exactive mass spectrometer, which was connected in-line with an LTQ-Orbitrap XL hybrid mass spectrometer equipped with an electrospray ionization source (Thermo Fisher Scientific). This instrument is located in the QB3/Chemistry Mass Spectrometry Facility at the University of California at Berkeley. The LC was equipped with a C18 analytical column (VivA C18: 150 mm length × 1.0 mm inner diameter, 5-μm particles; Restek) and a 100 μL sample loop. Solvent A was H2O + 0.1% formic acid and solvent B was MeCN + 0.1% formic acid (v/vol). The elution program consisted of isocratic conditions at 0% B for 3 min, a linear gradient to 35% B over 32 min, a linear gradient to 95% B over 0.1 min, isocratic conditions at 95% B for 4.9 min, a linear gradient to 0% B over 0.1 min, and isocratic conditions at 0% B for 19.9 min at a flow rate of 130 μL/min and 40 °C for the column compartment.

Full-scan mass spectra were acquired in the positive ion mode over the range of m/z = 100–1,000 using the Orbitrap mass analyzer, in profile mode for full-scan resolution setting of 0.02 (m/z = 200, full width at half-maximum peak height). MS/MS spectra of selected precursor ions were acquired using collision-induced dissociation in the positive ion mode using the linear ion trap, in centroid format, with the following parameters: isolation width 3 m/z units, normalized collision energy 30%, activation time 30 ms, and activation Q 0.25. Data acquisition and analysis were performed using Xcalibur software (version 2.0.7 SP1, Thermo).

RNA Isolation and RT-PCR. RNA was extracted from G. sulfurreducens PCA cell pellets using the bacterial RNA purification protocol of the Universal RNA Purification Kit (CHIMERx) with lysozyme and RB buffer from the kit. Quality of total RNA was checked on a 1% agarose gel. RNA was treated with RNase-free DNase (Promega), and then 1 μg total RNA was used for cDNA synthesis in a 20 μL reaction using iScript Select CDNA Synthesis Kit (BioRad) or AMV Reverse Transcriptase (New England Biolabs) with random hexamer primer. RT-PCR was performed using primers designed to span intergenic regions (SI Appendix, Table S4) and Taq DNA polymerase (New England Biolabs).

Live-Cell Imaging by Fluorescence Microscopy and Flow Cytometry. Constructs for testing the Gm0970-Spinach biosensors in vivo were created as described previously (14, 16) by inserting the aptamers into a tRNA scaffold encoded within the pET31b plasmid (SI Appendix, Table T1). Biosensors and cyclic dinucleotide cycler enzymes encoded in pET3a or pCRLA plasmids were coexpressed in E. coli BL21 (DE3) Star cells (Life Technologies). Fluorescence microscopy experiments and image analyses were carried out as described previously (14).

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Preparation of cell samples for flow cytometry was carried out in a similar manner to the microscopy procedure. Briefly, after inoculating fresh LB/Carb/Kan cultures from overnight starter cultures, cells were grown to an OD_{600} of ~0.5 and then induced with 1 mM IPTG at 37 °C for 2 h. Cells were pelleted at room temperature for 4 min at 3,700 rcf, washed once with M9 minimal media, and then resuspended in M9 minimal media containing 25 μM DFMHI to a final concentration of ~400 cells/μL based on OD_{600}. Cellular fluorescence was measured for 12,000–50,000 cells using either a BD Influx v7 cell sorter with BD FACSCanto Sortware (Version 1.0.6.650) or a Guava easyCyte BHT flow cytometer (Millipore) equipped with a 488-nm laser. The BD Influx v7 cell sorter is located in the Flow Cytometry Core Facilities at the University of California at Berkeley. Additional data are available in SI Appendix.

ACKNOWLEDGMENTS. We thank John D. Coates and members of his laboratory for advice on experiments with Geobacter. This work was supported in part by NIH Grant DP2 OD008677 (to M.C.H.), a Department of Defense Office of Science and Engineering Research Fellowship (to C.A.K.), a National Science Foundation graduate fellowship (to Z.F.H.), and NIH Training Grant T32 GM066698 (for Y.S. and T.F.B.). M.C.H. holds a Career Award at the Scientific Interface from the Burroughs Wellcome Fund.