Author Manuscript

# Riboswitches in eubacteria sense the second messenger c-di-AMP

**James W. Nelson**[1], **Narasimhan Sudarsan**[2,3], **Kazuhiro Furukawa**[3,*], **Zasha Weinberg**[2,3], **Joy X. Wang**[3,**], and **Ronald R. Breaker**[2,3,4,†]

[1]Department of Chemistry, Yale University, Box 208107, New Haven, CT 06520, USA

[2]Howard Hughes Medical Institute, Yale University, Box 208103, New Haven, CT 06520, USA

[3]Department of Molecular, Cellular and Developmental Biology, Yale University, Box 208103, New Haven, CT 06520, USA

[4]Department of Molecular Biophysics and Biochemistry, Yale University, Box 208103, New Haven, CT 06520, USA

## Abstract

Cyclic di-adenosine monophosphate (c-di-AMP) is a recently discovered bacterial second messenger implicated in the control of cell wall metabolism, osmotic stress responses, and sporulation. However, the mechanisms by which c-di-AMP triggers these physiological responses have remained largely unknown. Intriguingly, a candidate riboswitch class called *ydaO* associates with numerous genes involved in these same processes. Although a representative *ydaO* motif RNA recently was reported to weakly bind ATP, we report that numerous members of this noncoding RNA class selectively respond to c-di-AMP with sub-nanomolar affinity. Our findings resolve the mystery regarding the primary ligand for this extremely common riboswitch class and expose a major portion of the super-regulon of genes that are controlled by the widespread bacterial second messenger c-di-AMP.

A series of remarkable discoveries have been made recently in the area of bacterial second messengers[1,2]. One of these RNA-derived signaling compounds, c-di-GMP, is a cyclic dinucleotide made by fusing guanosine molecules via two 3′,5′-phosphodiester linkages. Fluctuations in local c-di-GMP concentrations in bacterial cells trigger a striking number of

fundamental changes in physiological status, and these changes are interpreted by the cellular machinery through binding of this second messenger to numerous protein[3,4] and RNA receptors[5,6].

A similar cyclic dinucleotide, c-di-AMP, was discovered in bacteria several years ago[7]. This compound, which consists of two adenosine nucleotides joined via two 3′,5′-phosphodiester linkages (Fig. 1a), has been implicated in signaling the presence of DNA damage[8] and cell wall stress[9,10]. Several protein receptors have recently been discovered that bind this molecule[11]. However, it is anticipated that numerous additional receptors remain to be discovered that sense and respond to changing concentrations of c-di-AMP. Since the discovery of c-di-AMP, we[12] and others[9] have considered the possibility that some newly-found riboswitch candidates might serve this purpose. As has occurred with other riboswitch classes[5,12], the discovery of a c-di-AMP responsive riboswitch would reveal much of the underlying biology controlled by this signaling molecule.

Nearly a decade ago, we reported the discovery of eight candidate riboswitch classes[13]. Four of these classes have since been proven to function as riboswitches for glycine[14], glucosamine-6-phosphate[15], 7-aminomethyl-7-deazaguanine (PreQ$_1$)[16], and divalent magnesium[17]. A fifth class called *ylbH* corresponds to the complementary sequence of a small riboregulator RNA called CsfG[18]. The three remaining "orphan" riboswitch classes are among the most common discovered to date, and are predicted to control fundamental and perhaps underappreciated aspects of bacterial physiology.

One of these three orphans, *ydaO*, is commonly associated with genes involved in cell wall metabolism, osmotic stress, and sporulation[13,19]. A search for all *ydaO* motif RNAs in bacterial DNA sequence databases revealed a total of 3012 representatives corresponding to a revised consensus sequence and secondary structure model (Fig. 1b). Given the number and the diversity of genes controlled by this riboswitch class[20], we expected that identification of its ligand would lead to new insights on how bacteria trigger cell wall remodeling and respond to extreme physicochemical stresses. Unfortunately, our previous attempts to identify the ligand were not successful[19], which highlights the challenge of identifying the natural ligand for some orphan riboswitch classes[21,22].

Recently, a *ydaO* motif representative from *Bacillus subtilis* was reported to sense and respond to ATP[23]. However, several existing observations suggested that ATP was not the primary ligand. First, we previously reported that certain mutations likely affecting energy metabolism and ATP production in *B. subtilis* result in an increase in *ydaO* motif-mediated gene expression[19]. However, the disruption of energy metabolism and the corresponding depletion of ATP also will affect the concentrations of numerous other metabolites. Therefore, these and similar observations[23] cannot be used as strong evidence for ATP riboswitch function. Second, our analyses had ruled out tight binding of ATP by a similar *ydaO* RNA construct[19]. Third, and perhaps most importantly, genes associated with *ydaO* motif RNAs do not implicate ATP as the biologically relevant ligand[13,19]. Fourth, our unpublished observations suggested that a much more tightly-binding ligand for this RNA existed in yeast extract.

Our further efforts to characterize this intriguing riboswitch candidate revealed that all members examined respond selectively to c-di-AMP, and strongly discriminate against ATP by more than one-million fold. Furthermore, *in vitro* and *in vivo* gene control experiments provide evidence that c-di-AMP, and not ATP, is the primary natural ligand for this riboswitch class. These findings reveal that riboswitch-mediated detection of c-di-AMP is important for the control of numerous genes involved in germination, peptidoglycan biosynthesis, and osmotic shock responses in a wide variety of bacteria.

## RESULTS

### The primary ligand for the *ydaO* riboswitch candidate

To establish the biologically relevant ligand for *ydaO* motif RNAs, we employed a biochemical purification strategy using yeast extract as a source of chemically diverse natural metabolites (see Methods). An assay for ligand binding was developed by combining equilibrium dialysis and in-line probing[24,25], and the putative aptamer portion of the *ydaO* mRNA of *Bacillus subtilis* called 165 *ydaO*[19] (Fig. 1c) was examined using this approach. By monitoring changes in the pattern of spontaneous RNA cleavage brought about by ligand binding (Supplementary Results, Supplementary Fig. 1a), we determined that a small amount of ligand or a close analog was present in this extract. Similarly, concentrations of the ligand were established in fractions generated during purification by reverse-phase liquid chromatography, and a sample enriched in the ligand was subjected to mass spectrum analysis (Supplementary Fig. 1b).

One of the major constituent compounds in this sample exhibited a mass and a fragmentation pattern indicative of adenosine monophosphate (AMP). Since we had previously tested and rejected 5′ AMP and several of its derivatives, including ATP, as possible biologically relevant ligands[19], we suspected that AMP might represent a breakdown product of the true ligand. Therefore, a wider collection of compounds containing this chemical moiety was examined for binding by the *ydaO* RNA (Supplementary Fig. 2). A pattern of spontaneous cleavage products that is identical to that observed with extract (Supplementary Fig. 1a) was observed when 165 *ydaO* was incubated with nanomolar amounts of c-di-AMP (Fig. 1d), or micromolar amounts of its linear derivatives pApA or ApA (Supplementary Fig. 2).

Sites exhibiting reduced spontaneous cleavage upon c-di-AMP addition largely reside in regions of sequence or structure conservation (Fig. 1c, Supplementary Fig. 3), indicating ligand-induced structural stabilization is occurring at these sites. In contrast, regions of constant cleavage reside in non-conserved loops. Similar results are observed with a second representative of this RNA class from *B. subtilis* called *yuaA* (Supplementary Fig. 4). These findings are consistent with the hypothesis that these RNAs are members of a class of riboswitches that sense and respond to c-di-AMP.

### Tight binding of c-di-AMP to *ydaO* motif RNAs

The 165 *ydaO* RNA structure exhibits half-maximal modulation when the c-di-AMP concentration is 700 pM during in-line probing (Figs. 1d and 1e). However, the binding

curve is steeper than that expected for 1-to-1 complex formation, suggesting that the dissociation constant ($K_D$) for the ligand might be below the concentration of radiolabeled RNA (~1 nM) used in this assay. Similar assays using a lower concentration of 165 *ydaO* RNA (Supplementary Fig. 5) or using a shortened RNA construct (Supplementary Fig. 6) suggests that a 1-to-1 binding complex is formed and that the apparent dissociation constant ($K_D$) is 100 pM or better. This affinity, which approaches that measured for a c-di-GMP-I riboswitch[26], is more than 100-fold better than what is needed for the RNA to sense a single ligand molecule present in the volume of a bacterial cell if the system were permitted enough time to reach equilibrium[22].

The selectivity of wild-type (WT) 165 *ydaO* RNA for c-di-AMP was assessed by establishing the $K_D$ values for a series of close analogs of this second messenger by using in-line probing. The linear analog pApA has a $K_D$ value of ~300 nM (Fig. 2a). Therefore, the riboswitch can discriminate against this natural breakdown product of c-di-AMP by more than 1,000 fold. Other naturally occurring and artificial analogs tested exhibit even weaker binding, with the exception of c-di-dAMP, the all-DNA version of the second messenger, and c-di-AMP$_{SS}$, a phosphorothioate-linked mimic of c-di-AMP which has potential utility as an inhibitor of c-di-AMP receptors (Supplementary Fig. 7). Replacement of phosphate with phosphorothioate in RNA linkages has been shown to render the analog less susceptible to phosphodiesterases[27], and is likely to improve the membrane permeability of the compound due to the enhanced lipophilicity of the sulfur atom relative to oxygen.

Notably, c-di-AMP binding affinity is more than six orders of magnitude greater than that observed for its immediate biosynthetic precursor, ATP[19], which recently has been claimed as the biologically relevant ligand for this riboswitch[23]. This large preference for c-di-AMP over ATP is also observed for *ydaO* aptamers from three other bacterial species of different phyla (Supplementary Figs. 8, 9, 10), which show no evidence of binding ATP even when tested at concentrations as high as 1 mM. Furthermore, by changing the $Mg^{2+}$ concentration and temperature of the in-line probing reaction to more closely approximate conditions found in the bacterial cytosol, we observed a complete loss of ATP binding (Supplementary Fig. 11). Under these same conditions, c-di-AMP is bound with a $K_D$ of ~10 nM.

A series of mutant 165 *ydaO* RNAs were examined to assess the importance of various conserved nucleotides or base-paired substructures (Fig. 2b). Mutations that alter strictly-conserved nucleotides (M1 and M2) or that perturb base-pairing (M3) disrupt c-di-AMP binding (Fig. 2c), while restoration of base-pairing (M4) restores binding. Similarly, a deletion that disrupts the formation of the predicted pseudoknot (M5) weakens the affinity of the riboswitch aptamer for c-di-AMP ($K_D$ ~20 nM) and eliminates ATP binding entirely (Fig. 2d). These findings are consistent with our hypothesis that the conserved sequences and secondary structure of this RNA class are necessary for c-di-AMP recognition.

## Regulation of gene expression by c-di-AMP riboswitches

The importance of this riboswitch class to gene regulation by c-di-AMP was evaluated *in vitro* by employing a transcription termination assay (Fig. 3a) using an RNA construct derived from the *yuaA* RNA of *B. subtilis*. Although concentrations of ATP as high as 3 mM were used, no substantive increase in transcription termination was observed beyond a

background level of termination that is typically observed with numerous other riboswitches[5,28,29]. In contrast, sub-micromolar amounts of c-di-AMP trigger increased transcription termination as predicted for this RNA construct, given that it tightly binds c-di-AMP and given that its expression platform architecture is consistent with that expected for a genetic "OFF" switch.

The role of this riboswitch class in gene regulation by c-di-AMP *in vivo* was assessed by employing a construct carrying the c-di-AMP riboswitch from the *B. subtilis ydaO* gene fused to the *lacZ* gene from *E. coli* (Fig. 3b). Initially, we evaluated constructs driven either by the native *ydaO* promoter or the *lysC* promoter[30] for expression in *B. subtilis* by introduction via plasmids into normal cells and into *disA* knock-out (KO) cells (Supplementary Fig. 12). The *disA* gene codes for one of three known diadenylate cyclase enzymes in *B. subtilis*[8]. With either promoter, increased expression was observed in the *disA* KO strain relative to the unaltered strain, suggesting that this change in expression is at least in part due to riboswitch-mediated regulation. This finding is consistent with the results of our *in vitro* transcription analysis (Fig. 3a), which was performed using the *lysC* promoter from *B. subtilis*. Increased reporter gene expression also occurs when a different diadenylate cyclase gene is deleted (*cdaA* KO; previously known as *ybbP*)[31], which is again consistent with our hypothesis that riboswitch-mediated activation of gene expression occurs as c-di-AMP levels decrease (Fig. 3c).

Mutations that disrupt a conserved nucleotide in the aptamer domain (M1, Fig. 2b) or that disrupt base-pairing of the predicted intrinsic terminator stem of the riboswitch expression platform (M6, Fig. 3b) also increase reporter gene expression driven by the native promoter. As expected, the M6 construct carrying a disrupted terminator stem yields the highest expression, and other constructs in various genetic backgrounds exhibit somewhat lower levels of expression than M6. As reported previously[19], mutations that disrupt base-pairing increase reporter gene expression whereas compensatory mutations that restore base-pairing also restore expression to near wild-type levels. All these results are consistent with our hypothesis that increases in c-di-AMP levels in cells will yield decreased expression of the *ydaO* gene, most likely by promoting the formation of an intrinsic terminator stem. However, the precise mechanism by which c-di-AMP binding regulates terminator formation has not yet been established.

## The c-di-AMP super-regulon as revealed by riboswitches

The genetic contexts of the many c-di-AMP riboswitches in bacteria reveal that various lineages exploit c-di-AMP signaling to control different biological processes, or at least different responses associated with the same biological processes. For example, Actinobacteria use c-di-AMP riboswitches to regulate cell wall metabolism almost exclusively, whereas Cyanobacteria use them to control the transport and synthesis of osmoprotectants (Fig. 4, Supplementary Table 1). Other genes associated with c-di-AMP riboswitches, such as *sleB*[32] and *pdaA*[33], have been linked to spore germination in species of Clostridia and Bacillales. The discovery of c-di-AMP riboswitches reveals numerous ways in which this second messenger, known to be intimately involved in sporulation dynamics[8], exerts its effects on this important biological process.

We also observed c-di-AMP riboswitches in a number of important contexts (Supplementary Dataset 1). Of particular interest is the *rpfA* gene of *Mycobacterium tuberculosis* and its homologs in many other organisms, which are commonly associated with a c-di-AMP riboswitch (Supplementary Figure 13). The *rpfA* gene, in conjunction with other resuscitation-promoting factors, is important for the transition of *M. tuberculosis* from stasis to growth during infection[34,35]. A role for c-di-AMP in this process has not previously been suggested. Additionally, we observe annotated GGDEF domains controlled by c-di-AMP riboswitches, suggesting that either c-di-AMP controls the synthesis of c-di-GMP in certain organisms or that these domains represent novel diadenylate cyclases. It is likely that the gene associations of c-di-AMP riboswitches will reveal additional novel roles for this second messenger.

## DISCUSSION

Since 2002, numerous riboswitches have been reported for a diverse collection of cellular metabolites and ions[22]. Typically, new candidate riboswitch classes are identified as conserved RNA motifs located in the 5′ untranslated regions of bacterial genes. Very often, the identity of these affiliated genes informs and directs the search for the riboswitch ligand, enabling their rapid validation and characterization. For certain riboswitch candidates, such as *ydaO, yybP*, and *ykkC*, these gene associations do not allow for the facile identification of a common signaling molecule[13,19,21]. Ligand discovery can be impeded by incomplete gene annotations, unrecognized connections between biological processes controlled by the riboswitch, or perhaps even discovery of the riboswitch class before its ligand is known to science.

Some of these orphan riboswitch candidates have resisted assignment of a ligand for more than a decade[13,19,21]. To date, the function of only four orphan riboswitch classes have successfully been resolved: c-di-AMP (as described herein), preQ$_1$-I[16,36,37], magnesium[17], and fluoride[12,38]. Our strategy for identifying the ligand for c-di-AMP riboswitches exploited the rich diversity of compounds in yeast extract, which contains either c-di-AMP, or a closely-related compound. However, other challenging orphan riboswitch candidates might require tailored strategies because some ligands might be unique to bacteria or are otherwise not present in an extract.

Recently, the same *ydaO* motif representative examined in our study was proposed to function as an ATP-sensing riboswitch[23]. Although we observe ATP binding at concentrations in the low millimolar range for both the 165 *ydaO* construct, and the shorter construct examined previously[23], no evidence for ATP binding was observed when subjecting the *yuaA* representative from *B. subtilis* to in-line probing, even when tested at 3 mM (Supplementary Figure 4). In contrast, this RNA (and all other representatives tested) exhibit high affinity for c-di-AMP. This exceptionally tight binding of c-di-AMP by numerous members of this riboswitch class is expected if this second messenger, and not ATP, is the primary functional ligand.

There are a number of additional observations that support this conclusion. For example, although the $K_D$ value of 165 *ydaO* RNA for ATP (as obtained under standard in-line

probing conditions) is similar to the cellular concentration of ATP, it is likely that many riboswitches operate as kinetically-driven, rather than thermodynamically-driven, genetic components[22,39-44]. Therefore, concentrations of ligand needed to trigger gene control under kinetic constraints might be orders of magnitude higher than the $K_D$ measured in the test tube under equilibrium conditions[23]. Indeed, in an in-line probing assay performed at 37°C with 10 mM $Mg^{2+}$ (conditions that more closely approximate that of the *in vivo* reporter assays), ATP binding is no longer apparent, while c-di-AMP binding is retained (Supplementary Figure 11). If the rate constant for ATP association with the riboswitch is poor, the concentration of ATP needed to trigger riboswitch function would need to be super-physiological.

Notably, a member of the *S*-adenosylhomocysteine (SAH) riboswitch class binds ATP with a $K_D$ of ~100 μM[45], which is even better than the affinity for ATP observed with the 165 *ydaO* RNA construct. However, the genetic contexts of SAH riboswitches, which are almost always associated with SAH hydrolase or other genes linked to *S*-adenosylmethionine (SAM) coenzyme biosynthesis,[46] strongly suggests that ATP is not the primary ligand for members of this riboswitch class. Similarly, the genes associated with *ydaO* RNAs strongly implicate c-di-AMP, and not ATP, as the biologically relevant ligand (Supplementary Table 1). For example, c-di-AMP riboswitches control *ktrA* and *kdpD* genes in numerous organisms. These genes code for proteins which were recently shown to directly bind c-di-AMP in *S. aureus*[11]. Many of the genes controlled by this riboswitch class are involved with cell wall metabolism and osmotic stress responses. Recently, these same processes were functionally linked to c-di-AMP signaling[9,11], which implicates this compound as the natural ligand for the riboswitch. Furthermore, we currently only find representatives of this riboswitch class among bacterial phyla known to carry genes coding for diadenylate cyclases, which are the proteins that synthesize c-di-AMP.

Results from a mutagenesis screen revealed that the inactivation of genes involved in energy metabolism alters expression of genes controlled by *ydaO* motif RNAs[19], and compounds that inhibit ATP synthesis also cause riboswitch-mediated changes in gene expression[23]. However, given the central role that ATP plays in metabolic processes, these observations are not sufficient to conclude that ATP is the primary ligand for this riboswitch class[19]. For example, the depletion of ATP might directly affect the production of c-di-AMP because this second messenger is synthesized from two ATP molecules[7].

The characteristics of the pseudoknot deletion construct (M5), which exhibits a loss of ATP binding as well as a substantial change in the structure near stems P5 and P7, suggest that ATP might be bound poorly by the WT 165 *ydaO* RNA in a site close to the P5-P6-P7 region. This site might normally serve as a binding site for one of the two adenosyl residues of c-di-AMP. If true, then the remainder of the highly-conserved sequences and structures of the riboswitch aptamer might be critical for forming the full binding pocket that strongly binds both adenosyl moieties of c-di-AMP. This data, combined with our observations that other representatives of this riboswitch class do not bind ATP at a concentration of 1 mM, suggests that the weak binding of ATP might not be biologically relevant. However we do not rule out the possibility that ATP can be bound by c-di-AMP riboswitches in some

species under certain cellular conditions, regardless of whether any resulting gene regulation is helpful or detrimental to the cell.

The c-di-AMP riboswitch class is among the first widely distributed biological receptors for this compound to be identified. Therefore, the distribution of c-di-AMP riboswitch representatives and their associated genes can be used to create a more comprehensive directory of organisms and biological pathways that employ c-di-AMP signaling. From these new data, it is apparent that c-di-AMP is involved in signaling key developmental processes in numerous species of bacteria. Our findings also lend support for the recently published conclusion that c-di-AMP controls responses to osmotic shock[11] in Firmicutes, Cyanobacteria, and Deltaproteobacteria, though not in Actinobacteria. Additionally, since riboswitch RNAs function as molecular sensors for their ligands, engineered constructs could be used to establish c-di-AMP levels in a variety of biological contexts, including during host infection by bacteria[47]. Such knowledge would empower microbiologists to more fully establish the wide-ranging affects of c-di-AMP on the fundamental processes controlled by this important second messenger.

## Methods

### Chemicals and reagents

Cyclic dinucleotides were purchased from Biolog Life Science Institute. Linear dinucleotides were purchased from Biolog and Oligos, Etc. [$\gamma$-$^{32}$P] ATP was purchased from Perkin Elmer and used within two weeks of receipt. All other fine chemicals were purchased from Sigma-Aldrich. Bulk chemicals were purchased from J.T. Baker, unless otherwise noted. Enzymes were purchased from New England Biolabs, unless otherwise noted. All solutions were prepared using deionized water (dH$_2$O) and either autoclaved or filter sterilized (using 0.22 μm filters, Millipore) prior to use, or were prepared using pre-sterilized components and used immediately.

### Purification of Yeast Extract

Purified yeast metabolite extracts were prepared by reverse-phase chromatographic separation of Bacto™ Yeast Extract (Becton, Dickinson and Company). 20 cc Sep-Pak C18 cartridges (Vac Cartridge, Waters) were washed first with 50% acetonitrile (80 mL) and then 50 mM triethylamine-acetate (TEAA; pH 7.0 at 23°C). Yeast extract powder (500 g) was dissolved in 3 L of 50 mM TEAA, 30 mL aliquots were loaded onto the cartridges, and the active fraction was recovered by elution with 5% acetonitrile. The presence of the ligand was monitored by in-line probing using the *B. subtilis* 165 *ydaO* RNA as described below.

Active samples were concentrated via rotary evaporation and the resulting solution purified via HPLC (Agilent Technologies 1200 series, 21.2 mm × 250 mm C18 column). Extracts were separated over a 0%-5.8% acetonitrile:TEAA gradient (5 mL min$^{-1}$ over 40 minutes) and the eluant was monitored via absorbance at 210, 254, and 320 nm. One-minute fractions were collected and again subjected to in-line probing to identify active fractions. After identifying the elution time of the modulating fraction, HPLC purification was repeated such that we obtained ~4 nmol of purified ligand in 200 μL from 500 g of yeast extract.

## Mass spectrometry of the active sample

Samples (20 μL) from concentrated active fractions and samples from inactive fractions (immediately adjacent to the active fractions) were dried under vacuum and resuspended in 40 μL of 2% aqueous methanol. The samples were then desalted using a C18 ZipTip (Millipore) and eluted with 40 μL of 50% aqueous methanol. For each sample, 5 μL was directly infused via Advion TriVersa NanoMate into a Bruker 9.4T FT-ICR MS. 100 scans (ionized in negative mode), each consisting of 1.0 second of ion accumulation time, were collected and summed. Analysis of the ligand-containing fraction revealed four peaks that were both concentrated enough for MS/MS analysis and enriched relative to their amounts in the adjacent inactive fractions. Each of these peaks was subjected to MS/MS, under similar conditions as described above, and the ion fragments collected and analyzed. Of the four peaks, only one had a mass to charge ratio ($m/z$) which corresponded to a known biological compound (adenosine monophosphate), identified using both the Madison Metabolomics Consortium Database (MMCD) and Kyoto Encyclopedia of Genes and Genomes (KEGG)[48-50].

## In-Line Probing Reactions

Templates for RNA transcription were assembled as described previously[25]. Briefy, DNA templates were assembled either via PCR[19] or via primer extension of overlapping synthetic DNAs using *Taq* polymerase. In-line probing assays were conducted using methods similar to those described previously[24,25]. Modified in-line probing reactions (lower $Mg^{2+}$ concentration, higher temperature, lower pH) were conducted for 16 hours at 37°C in mixtures containing 2 mM $MgCl_2$, 100 mM KCl, and 100 mM Tris-HCl (pH 8.3 at 23°C).

$K_D$ values were determined by performing in-line probing of a specific RNA construct and varying the ligand concentration. Bands undergoing ligand-mediated changes in intensity were quantified and the values were adjusted by subtracting background and normalized relative to a band that does not undergo ligand-mediated changes. The resulting values, termed fraction of RNA modulated, were scaled from 0 (minimum) to 1 (maximum) and plotted versus the logarithm of the molar concentration of ligand. Typically the maximum value was established by averaging the normalized signal observed for two or three concentrations of c-di-AMP that appear sufficient to fully modulate the structure of the riboswitch. The data were fit to a standard sigmoidal dose-response curve (constraining modulation with no ligand to 0 and full modulation to 1.0) to obtain apparent $K_D$ values.

## Transcription Termination Assays

Transcription termination assays were performed as described previously[5,6] with only minor modifications. DNA templates consisting of the *yuaA* riboswitch from *B. subtilis* (−238 to +30 relative to the translation start site) and the *lysC* promoter from *B. subtilis* were constructed by PCR using *B. subtilis* genomic DNA and a forward primer that contained an RNA polymerase promoter sequence, as described previously[30]. The construct was designed such that no C residue was encountered within the first 19 nucleotides from the transcription start site. Transcription was initiated by the addition of a mixture of UTP and ATP (2.5 μM each) and GTP (1 μM) and [α-$^{32}$P]-GTP (2 μCi) in transcription buffer (40 mM Tris-HCl [pH 7.5 at 23°C], 150 mM KCl, 10 mM $MgCl_2$, 0.01 mg mL$^{−1}$ BSA, 1% [v/v] glycerol, 4

pmol DNA template, and 0.045 U μL$^{-1}$ *E. coli* RNA polymerase (Epicenter). The mixture was incubated for 10 minutes at 37°C and halted complexes were restarted by the addition of 150 μM each of ATP, GTP, and CTP, and 50 μM UTP. The amount of possible ligand added to each transcription reaction is indicated for each experiment (either ATP, c-di-AMP, or c-di-GMP). Heparin (0.1 mg mL$^{-1}$) was simultaneously added to prevent RNA polymerase reinitiation. Each reaction was incubated for an additional 20 min at 37°C and the resulting products were analyzed by using denaturing 10% PAGE.

With the exception of the experiment testing varying concentrations of ligand, at least three replicates were conducted for each assay with essentially identical results, and the images shown are representative of these experiments. The amounts of full length and terminated transcripts were established by correcting for the different numbers of G residues in the RNA products. The percent of [α-$^{32}$P]-GTP compared to total GTP concentration in the initiation and elongation reactions (7% and 0.4%, respectively) was established, and the relative amount of radioactivity per terminated ($R_T$) and full length ($R_{FL}$) transcripts was calculated for each transcript as described previously[5,6].

### Construction of reporter constructs and genetic knockouts

To examine whether the riboswitches respond to c-di-AMP *in vivo*, we constructed a series of reporter constructs where β-galactosidase activity was used as a surrogate reporter for the level of activation of the riboswitch. Details are provided below.

**Bacterial strains and culture conditions**—*B. subtilis* strain PY79, its isogenic derivative strain YA5 (harbors the *disA* deletion *disA::tet*), and the vector pDG1661 were obtained from the *Bacillus* Genetic Stock Center (The Ohio State University). A *cdaA* deletion mutant derivative of strain PY79 was constructed by transforming a PCR fragment containing the *cdaA* ORF flanking regions interrupted by an erythromycin cassette derived from the plasmid pE194 as described previously[51]. Briefly, each flanking sequence was constructed via PCR amplification of the relevant sequence from genomic DNA (the primers contained an overlap sequence derived from pE194), while the erythromycin cassette was amplified from the above plasmid in three separate PCR reactions. Each PCR product was combined in sequential PCR reactions, and the resulting fragment cloned via TOPO-TA (Invitrogen) for transformation into TOP-10 cells (Invitrogen). Following transformation, the desired plasmid was confirmed via sequencing, and the fragment amplified using the appropriate primers and transformed into *B. subtilis* and integrated into the *cdaA* locus. Transformants were plated on erythromycin-containing media, single colonies picked, and cells were propagated in antibiotic-free media to ensure a stable integration had occurred. The deletion of *cdaA* was confirmed by sequencing the PCR product of the *cdaA* locus, including the flanking region.

*Bacillus* strains were grown routinely in LB or Nutrient Broth. When required, growth medium were supplemented with antibiotics at the following concentrations: erythromycin, 0.5 μg mL$^{-1}$; tetracycline, 5 μg mL$^{-1}$; chloramphenicol, 5 μg mL$^{-1}$.

**Reporter constructs**—The region between nucleotides −466 to −129 with respect to the *ydaO* translational start site in *B.* subtilis encompassing the *ydaO* riboswitch and native

promoter were amplified by PCR and cloned in to the vector pDG1661 as an *Eco*RI-*Bam*HI fragment to generate a transcriptional fusion with a *lacZ* reporter gene. The resulting construct was integrated in to the *amyE* locus of *B. subtilis* as described previously[30]. A reporter construct containing the *ydaO* riboswitch and a promoter for the *B. subtilis lysC* gene[30] was constructed in an identical way using a forward primer containing the promoter sequence. Reporter constructs containing mutations to the riboswitch aptamer or predicted intrinsic terminator were prepared by first performing two-step PCR using primers bearing the desired mutations to obtain the appropriate DNA construct, before ligation into pDG1661 as described above. Mutation and compensation mutation constructs were derived from constructs described previously[5] and prepared using appropriate PCR primers.

A pseudo-translational reporter to monitor *ydaO* riboswitch regulation (as used in Supplementary Fig. 12) was generated in an identical way to that described above, except that the region between nucleotides −466 and +32 with respect to the *ydaO* translational start site in *B. subtilis* was amplified.

### Reporter assays

Following transformation of *B. subtilis* cells with the appropriate genetic background with the desired reporter, a single bacterial colony was used to inoculate 2 mL of either Nutrient Broth (NB) or Lysogeny Broth (supplemented with 5 μg mL$^{-1}$ chloramphenicol) in a 14 mL sterile tube and allowed to grow with vigorous shaking for 20 h at 37°C. The resulting cultures were vortexed immediately before transferring 80 μL aliquots into the wells of a black, clear-bottom, 96-well plate. Absorbance at 595 nm was measured using a Tecan Synergy 2 plate reader. Then, 80 μL of modified Z-buffer (60 mM $Na_2HPO_4$, 40 mM $NaH_2PO_4$, 10 mM KCl, 1 mM $MgSO_4$) was added to each well, followed by 40 μL of a 1 mg mL$^{-1}$ solution of 4-methylumbelliferyl-β-ᴅ-galactopyranoside (dissolved in 50:50 v/v DMSO:dH$_2$O), with care to ensure even mixing. The plate was allowed to stand at room temperature for 15 min, at which time 40 μL of 1 M $Na_2CO_3$ was added to halt the reaction. The resulting fluorescence (ex 360, em 460) was measured using the same plate reader. Media alone was used as a blank. Reporter gene expression values were calculated via the following equation:

$$Gene\quad Expression\quad (Fln\quad Units) = \frac{Fluorescence - Fluorescence\,(blank)}{Time\,(min)\quad *\quad (Abs._{595} - Abs._{595}\quad (blank))}$$

This procedure is a variation of that published previously[52].

### RNA homology searches

To identify homologs of c-di-AMP riboswitches, we began with the previously established *ydaO/yuaA* motif alignment[53]. We conducted iterative rounds of search and alignment of newly found homologs using infernal versions 1.0 and 1.1[54]. Pseudoknots and some other features were aligned manually. Variable-length hairpins corresponding to stems P2, P4 and P6 were re-aligned using the MFE prediction of the RNAfold program[55], when that structure was a plausible hairpin, or via manual alignment. Little attempt was made to correctly align

all nucleotides in these regions or to fully distinguish between variants that might contain a multistem structure where a hairpin structure is normal.

The set of genomic sequences searched consisted of those downloaded from RefSeq version 44[56], and the Human Microbiome Project[57], IMG/M[58], MG-RAST[59], CAMERA[60] and GenBank[61]. Gene predictions were obtained as previously described[62], and conserved domains within the predicted protein products were determined using version 2.25 of the Conserved Domain Database as described previously[12].

### Genes regulated by c-di-AMP riboswitches

Our approach to elucidating the c-di-AMP regulon was based on an earlier procedure[12]. Genes were predicted to be regulated by a c-di-AMP riboswitch when they were within 700 base pairs of that riboswitch and transcribed on the same DNA strand. Additional downstream genes were predicted to reside in a regulated operon until a gene was found that was encoded on the opposite DNA strand, or was more than 100 base pairs from the end of the preceding gene.

Categories in Fig. 4 (*e.g.*, "Cell Wall Metabolism") were conceived based on a qualitative evaluation of the annotated functions of conserved domains in regulated genes. Conserved domains were assigned to categories as appropriate. The assignment of conserved domains to categories is given in Supplementary Table 1. Raw frequencies were calculated as the fraction of regulated operons containing at least one domain within the given category. We selected a per-operon fraction rather than a per-gene fraction, because many categories involve multiple co-transcribed genes. With a per-gene fraction, categories that happen to encompass many domains would have inflated frequencies. When sequence databases represent multiple organisms within the same or closely related species, the properties of these organisms can be artificially emphasized. Therefore, we derive multiplicative weights for c-di-AMP riboswitches by applying Infernal's[54] implementation of the GSC algorithm[63]. We then apply each riboswitch's weight to the operon it regulates.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgements

## References

1. Römling U. Great times for small molecules: c-di-AMP, a second messenger candidate in bacteria and archaea. Sci. Signal. 2008; 1:pe39. [PubMed: 18714086]

2. Pesavento C, Hengge R. Bacterial nucleotide-based second messengers. Curr. Opin. Microbiol. 2009; 12:170–176. [PubMed: 19318291]

3. Sondermann H, Shikuma NJ, Yildiz FH. You've come a long way: c-di-GMP signaling. Curr. Opin. Microbiol. 2012; 15:140–146. [PubMed: 22226607]

4. Krasteva PV, Giglio KM, Sondermann H. Sensing the messenger: the diverse ways that bacteria signal through c-di-GMP. Prot. Sci. 2012; 21:929–948.

5. Sudarsan N, et al. Riboswitches in eubacteria sense the second messenger c-di-GMP. Science. 2008; 321:411–413. [PubMed: 18635805]

6. Lee ER, Baker JL, Weinberg Z, Sudarsan N, Breaker RR. An allosteric self-splicing ribozyme triggered by a bacterial second messenger. Science. 2010; 329:845–848. [PubMed: 20705859]

7. Witte G, Hartung S, Buttner K, Hopfner KP. Structural biochemistry of a bacterial checkpoint protein reveals diadenylate cyclase activity regulated by DNA recombination intermediates. Mol. Cell. 2008; 30:167–178. [PubMed: 18439896]

8. Oppenheimer-Shaanan Y, Wexselblatt E, Katzhendler J, Yavin E, Ben-Yehuda S. c-di-AMP reports DNA integrity during sporulation in *Bacillus subtilis*. EMBO Rep. 2011; 12:594–601. [PubMed: 21566650]

9. Corrigan RM, Abbott JC, Burhenne H, Kaever V, Gründling A. c-di-AMP is a new second messenger in *Staphylococcus* aureus with a role in controlling cell size and envelope stress. PLoS Pathog. 2011; 7:e1002217. [PubMed: 21909268]

10. Smith WM, et al. Heat resistance and salt hypersensitivity in *Lactococcus lactis* due to spontaneous mutation of *limg_1816* (*gdpP*) induced by high temperature growth. Appl. Environ. Microbiol. 2012; 78:7753–7759. [PubMed: 22923415]

11. Corrigan RM, et al. Systematic identification of conserved bacteria c-di-AMP receptor proteins. Proc. Natl. Acad. Sci. USA. 2013; 110:9084–9089. [PubMed: 23671116]

12. Baker JL, et al. Widespread genetic switches and toxicity resistance proteins for fluoride. Science. 2012; 335:233–235. [PubMed: 22194412]

13. Barrick JE, et al. New RNA motifs suggest an expanded scope for riboswitches in bacterial genetic control. Proc. Natl. Acad. Sci. USA. 2004; 101:6421–6426. [PubMed: 15096624]

14. Mandal M, et al. A glycine-dependent riboswitch that uses cooperative binding to control gene expression. Science. 2004; 306:275–279. [PubMed: 15472076]

15. Winkler WC, Nahvi A, Roth A, Collins JA, Breaker RR. Control of gene expression by a natural metabolite-responsive ribozyme. Nature. 2004; 428:281–286. [PubMed: 15029187]

16. Roth A, et al. A riboswitch selective for the queuosine precursor preQ1 contans an unusually small aptamer domain. Nat. Struct. Mol. Biol. 2007; 14:308–317. [PubMed: 17384645]

17. Dann CE III, et al. Structure and mechanism of a metal-sensing regulatory RNA. Cell. 2007; 130:878–892. [PubMed: 17803910]

18. Marchais A, Duperrier S, Durand S, Gautheret D, Stragier P. CsfG, a sporulation-specific, small non-coding RNA highly conserved in endospore formers. RNA Biol. 2011; 8:358–364. [PubMed: 21532344]

19. Block KF, Hammond MC, Breaker RR. Evidence for widespread gene control function by the *ydaO* riboswitch candidate. J. Bacteriol. 2010; 192:3983–3989. [PubMed: 20511502]

20. Breaker RR. Riboswitches and the RNA world. Cold Spring Harb. Perspect. Biol. 2012; 4:a003566. [PubMed: 21106649]

21. Meyer MM, et al. Challenges of ligand identification for riboswitch candidates. RNA Biol. 2011; 8:5–10. [PubMed: 21317561]

22. Breaker RR. Prospects for riboswitch discovery and analysis. Mol. Cell. 2011; 43:867–879. [PubMed: 21925376]

23. Watson PY, Fedor MJ. The *ydaO* motif is an ATP-sensing riboswitch in *Bacillus subtilis*. Nat. Chem. Biol. 2012; 8:963–965. [PubMed: 23086297]

24. Soukup GA, Breaker RR. Relationship between internucleotide linkage geometry and the stability of RNA. RNA. 1999; 5:1308–1325. [PubMed: 10573122]

25. Regulski EE, Breaker RR. In-line probing analysis of riboswitches. Methods Mol. Biol. 2008; 419:53–67. [PubMed: 18369975]

26. Smith KD, et al. Structural basis of ligand binding by a c-di-GMP riboswitch. Nat. Struct. Mol. Biol. 2009; 16:1218–1223. [PubMed: 19898477]

27. Shanahan CA, Gaffney BL, Jones RA, Strobel SA. Identification of c-di-GMP derivatives resistance to an EAL domain phosphodiesterase. Biochemistry. 2012; 2013:365–377.

28. Murphy McDaniel BA, Grundy FJ, Artsimovitch I, Henkin TM. Transcription termination control of the S box system: Direct measurement of *S*-adenosylmethionine by the leader RNA. Proc. Natl. Acad. Sci. USA. 2003; 100:3083–3088. [PubMed: 12626738]

29. Epshtein V, Mironov AS, Nudler E. The riboswitch-mediated control of sulfur metabolism in bacteria. Proc. Natl. Acad. Sci. USA. 2003; 100:5052–5056. [PubMed: 12702767]

30. Sudarsan N, Wickiser JK, Nakamura S, Ebert MS, Breaker RR. An mRNA structure in bacteria that controls gene expression by binding lysine. Genes Dev. 2003; 17:2688–2697. [PubMed: 14597663]

31. Mehne FM, et al. Cyclic di-AMP homeostasis in *Bacillus subtilis*; both lack and high level accumulation of the nucleotide are detrimental for cell growth. J. Biol. Chem. 2013; 288:2004–2017. [PubMed: 23192352]

32. Moriyama R, et al. Expression of a germination-specific amidase, SleB, of *Bacilli* in the forespore compartment of sporulating cells and its localization on the exterior side of the cortex in dormant spores. J. Bacteriol. 1999; 181:2373–2378. [PubMed: 10197998]

33. Gilmore ME, Bandyopadhyay D, Dean AM, Linnstaedt SD, Popham DL. Production of muramic δ-lactam in *Bacillus subtilis* spore peptidoglycan. J. Bacteriol. 2004; 186:80–89. [PubMed: 14679227]

34. Kana BD, et al. The resuscitation-promoting factors of *Mycobacterium tuberculosis* are required for virulence and resuscitation from dormancy but are collectively dispensable for growth *in vitro*. Mol. Microbiol. 2008; 67:672–684. [PubMed: 18186793]

35. Kana BD, Mizrahi V. Resuscitation-promoting factors as lytic enzymes for bacterial growth and signaling. Pathog. Dis. 2010; 58:39–50.

36. Klein DJ, Edwards TE, Ferré-D'Amaré AR. Cocrystal structure of a class I $preQ_1$ riboswitch reveals a pseduoknot recognizing an essential hypermodified nucleobase. Nat. Struct. Mol. Biol. 2009; 16:343–344. [PubMed: 19234468]

37. Kang M, Peterson R, Feigon J. Structural insights into riboswitch control of the biosynthesis of queuosine, a modified nucleotide found in the anticodon of tRNA. Mol. Cell. 2009; 33:784–790. [PubMed: 19285444]

38. Ren A, Rajashankar KR, Patel DJ. Fluoride ion encapsulation by $Mg^{2+}$ ions and phosphates in a fluoride riboswitch. Nature. 2012; 486:85–89. [PubMed: 22678284]

39. Wickiser JK, Winkler WC, Breaker RR, Crothers DM. The speed of RNA transcription and metabolite binding kinetics operate an FMN riboswitch. Mol. Cell. 2005; 18:49–60. [PubMed: 15808508]

40. Wickiser JK, Cheah MT, Breaker RR, Crothers DM. The kinetics of ligand binding by an adenosine-sensing riboswitch. Biochemistry. 2005; 44:13404–13414. [PubMed: 16201765]

41. Gilbert SD, Stoddard CD, Wise SJ, Batey RT. Thermodynamic and kinetic characterization of ligand binding to the purine riboswitch aptamer domain. J. Mol. Biol. 2006; 359:754–768. [PubMed: 16650860]

42. Lemay J-F, et al. Comparative study between transcriptionally- and translationally-acting adenine riboswitches reveals key differences in riboswitch regulatory mechanisms. PLoS Genet. 2011; 7:e1001278. [PubMed: 21283784]

43. Haller A, Soulière MF, Micura R. The dynamic nature of RNA as key to understanding riboswitch mechanisms. Acc. Chem. Res. 2011; 44:1339–1348. [PubMed: 21678902]

44. Frieda KL, Block SM. Direct observation of cotranscriptional folding in an adenine riboswitch. Science. 2012; 338:397–400. [PubMed: 23087247]

45. Edwards AL, Reyes FE, Héroux A, Batey RT. Structural basis for recognition of *S*-adenylhomocysteine by riboswitches. RNA. 2010; 16:2144–2155. [PubMed: 20864509]

46. Wang JX, Lee ER, Morales DR, Lim J, Breaker RR. Riboswitches that sense *S*-adenylhomocysteine and activate genes involved in coenzyme recycling. Mol. Cell. 2008; 29:691–702. [PubMed: 18374645]

47. Woodward JJ, Iavarone AT, Portnoy DA. c-di-AMP secreted by intracellular *Listeria monocytogenes* activates a host type I interferon response. Science. 2010; 328:1703–1705. [PubMed: 20508090]

48. Markley JL, et al. New bioinformatics resources for metabolomics. Pac. Symp. Biocomput. 2007:157–168. [PubMed: 17990489]

49. Kanehisa M, Goto S, Sato Y, Furumichi M, Tanabe M. KEGG for integration and interpretation of large-scale molecular datasets. Nucleic Acids Res. 2012; 40:D109–D114. [PubMed: 22080510]

50. Kanehisa M, Goto S. KEGG: Kyoto Encyclopedia of Genes and Genomes. Nucleic Acids Res. 2000; 28:27–30. [PubMed: 10592173]

51. Wach A. PCR-Synthesis of marker cassettes with long flanking homology regions for gene disruptions in *S. cerevisiae*. Yeast. 1996; 12:259–265. [PubMed: 8904338]

52. Vidal-Aroca F, et al. One-step high-throughput assay for quantitative detection of β-galactosidase activity in intact Gram-negative bacteria, yeast, and mammalian cells. BioTechniques. 2006; 40:433–400. [PubMed: 16629389]

53. Barrick JE, Breaker RR. The distributions, mechanisms, and structures of metabolite-binding riboswitches. Genome Biol. 2007; 8:R239. [PubMed: 17997835]

54. Nawrocki EP, Kolbe DL, Eddy SR. Infernal 1.0: inference of RNA alignments. Bioinformatics. 2009; 25:1335–1337. [PubMed: 19307242]

55. Hofacker, IL. Current Protocols in Bioinformatics. Boyle, A., editor. John Wiley and Sons, Inc.; 2009. p. 12.2.1-12.2.16.

56. Pruitt KD, Tatusova T, Maglott DR. NCBI reference sequences (RefSeq): a curated non-redundant sequence database of genomes, transcripts, and proteins. Nucleic Acids Res. 2007; 35:D61–D65. [PubMed: 17130148]

57. Gevers D, et al. The human microbiome project: a community resource for the healthy human microbiome. PLoS Biol. 2012; 10:e1001377. [PubMed: 22904687]

58. Markowitz VM, et al. IMG/M: a data management and analysis system for metagenomes. Nucleic Acids Res. 2008; 36:D534–D538. [PubMed: 17932063]

59. Meyer F, et al. The metagenomics RAST server – a public resource for the automatic phylogenetic and functional analysis of metagenomes. BMC Bioinformatics. 2008; 9:386–393. [PubMed: 18803844]

60. Sun S, et al. Community cyberinfrastructure for advanced microbial ecology research and analysis: the CAMERA resource. Nucleic Acids Res. 2011; 39:D546–D551. [PubMed: 21045053]

61. Benson DA, Karsch-Mizrachi I, Lipman DJ, Ostell J, Wheeler DL. Genbank. Nucleic Acids Res. 2008; 36:D25–DD30. [PubMed: 18073190]

62. Roth A, et al. A novel class of self-cleaving ribozymes is present in many species of bacteria and eukarya. Nature Chem. Biol. in press.

63. Gerstein M, Sonnhammer ELL, Chothia C. Volume changes on protein evolution. J. Mol. Biol. 1994; 236:1067–1078. [PubMed: 8120887]
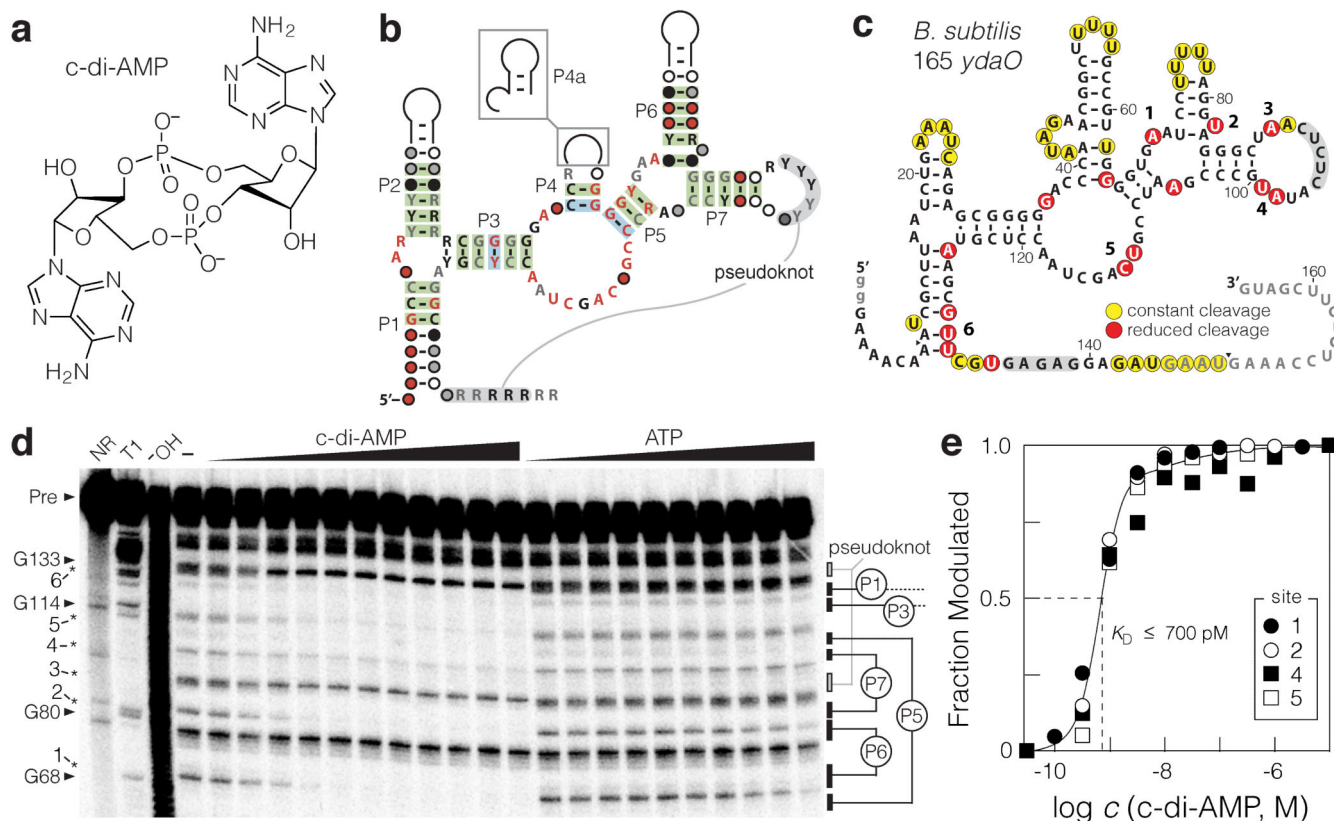
**Figure 1. Binding of c-di-AMP by a *ydaO* motif RNA**
**a,** Chemical structure of c-di-AMP. **b,** Consensus sequence and secondary structure of *ydaO* motif RNAs derived from ~ 3,000 examples. Red, black and gray nucleotides, respectively, are present in greater than 97, 90 and 75% of the representatives. Predicted base-paired substructures are labeled P1 through P7, with one pseudoknot as indicated. Green shading indicates phylogenetic evidence of base pairing. Other annotations are as described previously[19]. **c,** Structural modulation of the WT 165 *ydaO* RNA from *B. subtilis*. Highlighted nucleotides indicate locations of changes in spontaneous cleavage upon addition of c-di-AMP, mapped using the data in **d** and Supplementary Fig. 3. **d,** Polyacrylamide gel electrophoresis (PAGE) analysis of an in-line probing assay with 165 *ydaO* RNA exposed to various concentrations of c-di-AMP (100 pM to 10 μM in half-log intervals) or ATP (17.8 μM to 3.16 mM in quarter-log intervals). NR, T1 and ⁻OH, respectively, designate no reaction, partial digestion with either RNase T1 (cleaves after guanosine nucleotides) or hydroxide ions (cleaves after any nucleotide). Precursor RNA (Pre) and certain RNase T1 cleavage product bands are identified. Locations of spontaneous RNA cleavage changes brought about by c-di-AMP (regions 1 through 6) are identified by asterisks (see Supplementary Fig. 14 for the full length gel). **e,** Plot of the fraction of riboswitch RNA bound to ligand versus the logarithm of the molar concentration of c-di-AMP as inferred from the modulation of spontaneous cleavage products in **d**.
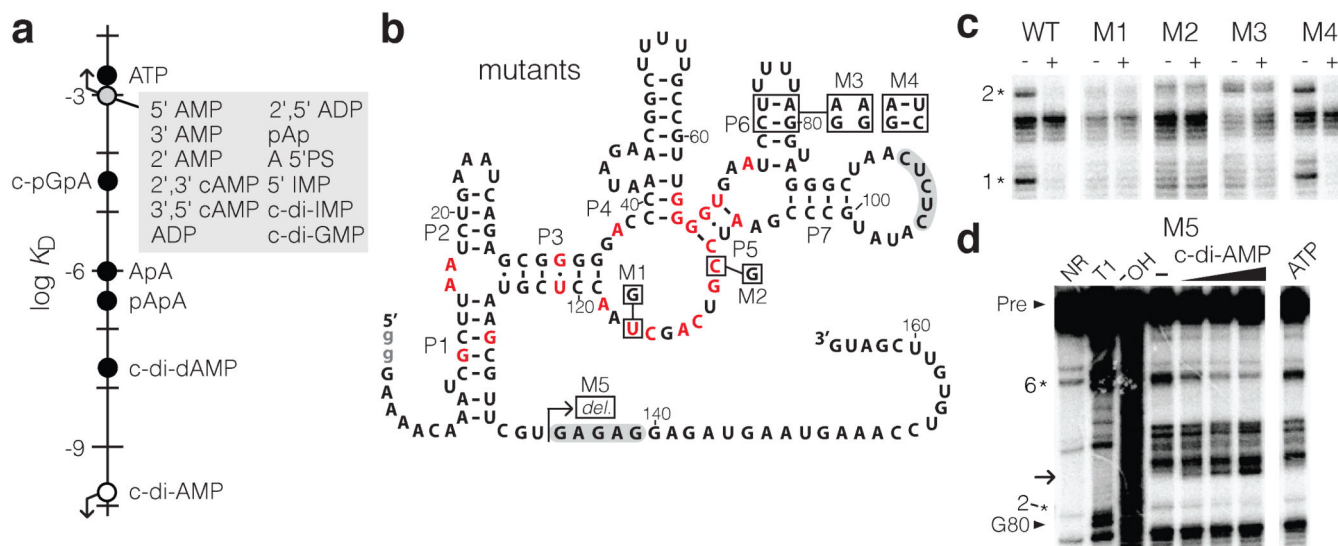
**Figure 2. Ligand binding characteristics of WT and variant 165 *ydaO* RNAs**

**a,** Plot of the $K_D$ values for the binding of c-di-AMP analogs by WT 165 *ydaO* RNA. A 5′PS is adenosine 5′-phosphosulfate, IMP is inosine 5′-phosphate, c-pGpA is the cyclic dinucleotide composed of one GMP and one AMP (also called cGA), and c-di-dAMP is the DNA analog of c-di-AMP. Gray circle and arrow indicates that the compounds listed exhibit no detectable binding at this concentration. Open circle and arrow indicates the $K_D$ for c-di-AMP is no poorer than this value. **b,** Various mutant constructs examined for ligand binding. Nucleotide changes made at the sites annotated M1 through M5 are boxed. Red letters identify highly conserved nucleotides. **c,** In-line probing analyses of WT and M1 through M4 RNAs as defined in b in the absence (−) or presence of 10 nM c-di-AMP. Gel images depict spontaneous RNA cleavage patterns encompassing sites 1 and 2 as defined in Fig. 1c (see Supplementary Fig. 15 for the full length gel). **d,** In-line probing analysis of M5 in the absence (−) of ligand, or the presence of c-di-AMP (10, 32 or 100 nM), or ATP (1 mM). Gel images depict the region encompassing sites 1 through 6 with annotations as described for Fig. 1d. The arrow identifies nucleotides in the loop region of P7 that become unstructured on ligand binding, as expected since their complementary sequences have been removed in this construct (see Supplementary Fig. 16 for the full length gel).
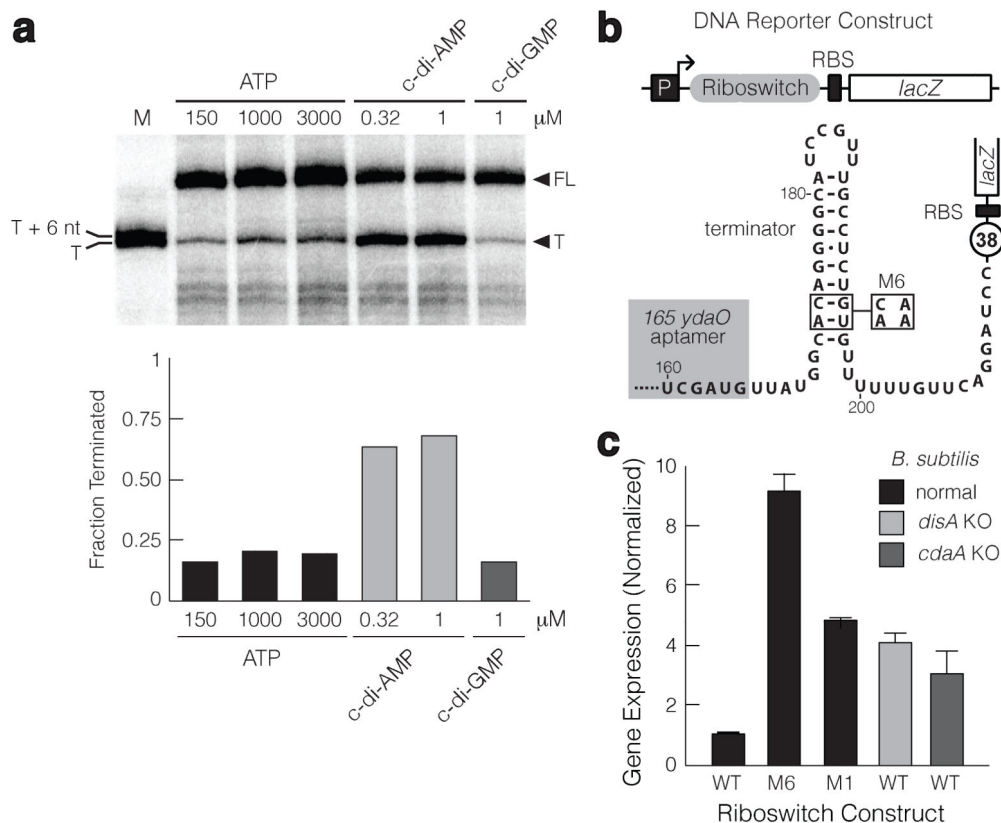
**Figure 3. Riboswitch regulation of gene expression by c-di-AMP**

**a**, PAGE analysis of an *in vitro* transcription termination assay using the *yuaA* riboswitch from *B. subtilis*. T is the riboswitch-terminated RNA transcript and FL is the full-length run-off transcript. M is a marker lane comprising the transcription products from a similar DNA template encoding the riboswitch plus six additional nucleotides beyond the predicted terminator site (see Supplementary Fig. 17 for the full length gel). **b,** Reporter gene construct used to assess regulation by c-di-AMP riboswitches. P is the native *ydaO* promoter, *lacZ* is the reporter gene, and RBS is the ribosome binding site. The predicted intrinsic transcription terminator stem for the *B. subtilis ydaO* riboswitch is shown in detail. **c,** Plot of reporter gene expression for various riboswitch constructs and genetic backgrounds (normal is the YP79 strain of *B. subtilis*) normalized to the level of expression observed with the WT riboswitch construct. Error bars represent the standard deviation of three replicate experiments conducted on three different days.
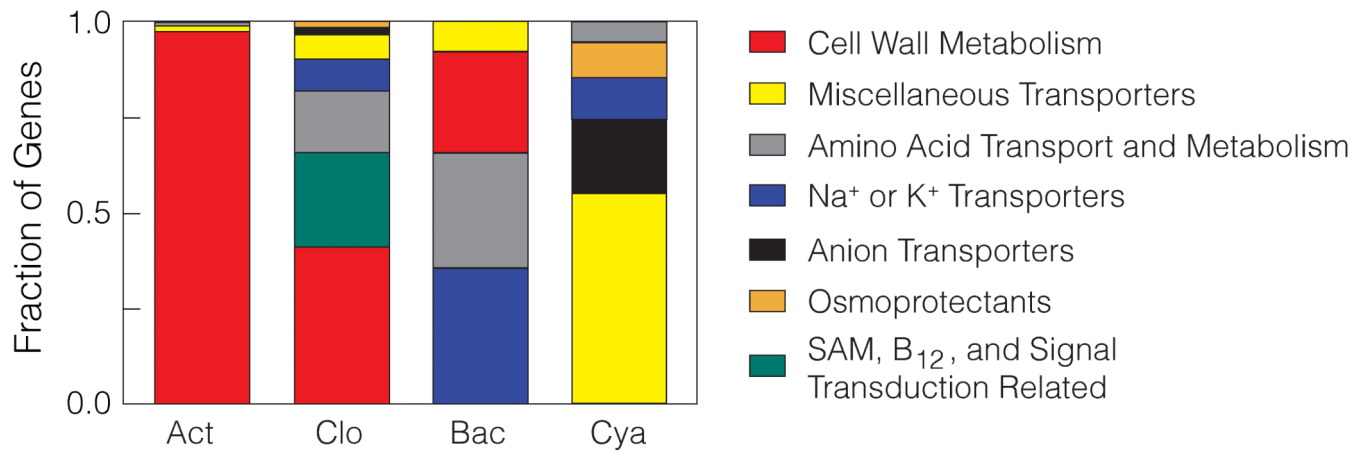
**Figure 4. The super-regulon for second messenger signaling through c-di-AMP riboswitches**
Bacterial lineages presented are Actinobacteria (Act), Bacillales (Bac), Clostridia, (Clo) and Cyanobacteria (Cya).