MICROBIOLOGY

A direct screen for c-di-GMP modulators reveals a *Salmonella* Typhimurium periplasmic L-arginine–sensing pathway

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Cyclic-di-GMP (c-di-GMP) is a bacterial second messenger that transduces internal and external signals and regulates bacterial motility and biofilm formation. Some organisms encode more than 100 c-di-GMP–modulating enzymes, but only for a few has a signal been defined that modulates their activity. We developed and applied a high-throughput, real-time flow cytometry method that uses a fluorescence resonance energy transfer (FRET)–based biosensor of free c-di-GMP to screen for signals that modulate its concentration within *Salmonella* Typhimurium. We identified multiple compounds, including glucose, *N*-acetyl-D-glucosamine, salicylic acid, and L-arginine, that modulated the FRET signal and therefore the free c-di-GMP concentration. By screening a library of mutants, we identified proteins required for the c-di-GMP response to each compound. Furthermore, low micromolar concentrations of L-arginine induced a rapid translation-independent increase in c-di-GMP concentrations and c-di-GMP–dependent cellulose synthesis, responses that required the regulatory periplasmic domain of the diguanylate cyclase STM1987. L-Arginine signaling also required the periplasmic putative L-arginine–binding protein ArtI, implying that L-arginine sensing occurred in the periplasm. Among the 20 commonly used amino acids, *S.* Typhimurium specifically responded to L-arginine with an increase in c-di-GMP, suggesting that L-arginine may serve as a signal during *S.* Typhimurium infection. Our results demonstrate that a second-messenger biosensor can be used to identify environmental signals and define pathways that alter microbial behavior.

INTRODUCTION

Bacterial sensing of environmental change is crucial for adaptation to a variety of niches. Hundreds of signal transduction proteins are encoded by bacterial genomes, but few specific signals and their roles in regulatory pathways are fully defined (*1*, *2*). Although many signal transduction proteins with defined signals regulate transcription, the sensing domains of these proteins can also be coupled to enzymatic control of second-messenger concentrations to more rapidly adapt to environments than signals that engage the transcription and translation machineries. Cyclic-di-GMP (c-di-GMP) is a bacterial nucleotide-based second messenger involved in sessile-to-motile transitions. Many species have dozens of c-di-GMP–metabolizing enzymes (CMEs), including diguanylate cyclases (DGCs) that synthesize c-di-GMP from two GTP (guanosine 5′-triphosphate) molecules and phosphodiesterases (PDEs) that degrade c-di-GMP (*1*, *3*, *4*). Downstream effectors that bind c-di-GMP control a number of bacterial behaviors, including flagellar-based motility, synthesis of extracellular carbohydrates that contribute to biofilm formation, inducible antibiotic resistance, and cell cycle progression (*1*, *3*, *4*). Some bacterial genomes encode more than a hundred CMEs (www.ncbi.nlm.nih.gov/Complete_Genomes/c-di-GMP.html). Many of these CMEs are proteins with putative sensing domains that may function in a signal transduction pathway. Screening for alteration of c-di-GMP concentration in real time will enable the identification of important signals and their sensory pathways through which c-di-GMP concentration is controlled.

Salmonella enterica serovar Typhimurium encodes 17 putative CMEs, among which 9 of these proteins contain periplasmic domains coupled through a transmembrane (TM) helix to cytoplasmic enzymatic domains (coupled domains), and another 6 of which contain six to eight TM domains linked to cytoplasmic enzymatic domains, such as MASE1 domains (*5*, *6*). Proteins with these properties may be involved in periplasmic sensing of small molecules. Periplasmic sensing of small molecules by methyl-accepting chemotaxis proteins (MCPs), histidine kinases (HKs), and CMEs enables the bacteria to monitor the periplasmic or extracellular space. Activation in response to an environmental signal can result from direct binding of small molecules to coupled domains. One such sensing module is the Cache1 domain, a periplasmic domain coupled to various signal transduction domains in proteins such as MCPs, HKs, and CMEs (*7*). Proteins with a Cache1 domain become activated after directly binding small molecules such as pyruvate, L-serine, L-arginine, L-asparagine, or L-proline (*8*, *9*). Further complexity arises when a periplasmic binding protein (PBP) binds the small molecule and then interacts with a coupled domain to activate the catalytic function of the protein. Multiple examples for HKs (*10*, *11*), MCPs (*12*–*14*), and CMEs (*15*) that require PBPs for activation are known. In addition, some coupled domains sense multiple signals either by interacting with multiple PBPs (*12*) or by both interacting with a PBP and directly binding a different small molecule (*13*).

Salmonella is a major cause of food poisoning estimated by the U.S. Centers for Disease Control and Prevention to cause 1.2 million illnesses per year in the United States alone. Its ability to form biofilms is especially important in the food-processing industry because *Salmonella* biofilms can form on many abiotic surfaces, and biofilm formation enhances this organism's resistance and persistence (*16*). *S.* Typhimurium is one of the two most common serovars in the United States and is a model organism for understanding the role of signaling in *Salmonella* infections. Only a few signals including oxygen, nitric oxide, and membrane damage have been reported to modulate c-di-GMP concentrations in these bacteria (*17*–*19*), but for most of these signals, neither has direct modulation of c-di-GMP concentrations nor a sensing pathway been identified. Therefore, we developed a mechanism to screen for changes in c-di-GMP

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concentrations in a real-time, high-throughput manner to identify potential environmental signals that regulate c-di-GMP concentration, as well as the CME and sensing pathways involved. Traditional methods for studying changes in c-di-GMP concentrations are limited by requiring cytoplasmic extraction and complex procedures to quantify nucleotide fractions that reflect total cellular second messenger and not the free concentration, which may result in aberrant results due to isolation of bound c-di-GMP. Alternatively, indirect determinations of c-di-GMP concentrations using phenotypic assays or transcription-based genetic reporters cannot monitor the dynamic changes in c-di-GMP concentrations over time and only report endpoint concentrations (*1*). We previously designed a fluorescence resonance energy transfer (FRET)–based c-di-GMP biosensor, which we refer to herein as "biosensor," based on the c-di-GMP–binding protein YcgR that detects free c-di-GMP concentrations (*20*). Here, we inserted the biosensor into *S.* Typhimurium and used flow cytometry to screen for signals that altered the amount of free c-di-GMP. Several compounds rapidly altered the amount of free c-di-GMP, including glucose, *N*-acetyl-D-glucosamine (GlcNAc), salicylic acid, and L-arginine. Analysis of *S.* Typhimurium mutants enabled the identification of different CMEs required for the response to different compounds. Furthermore, we identified a potential model of L-arginine sensing that requires ArtI (a putative L-arginine PBP) and the DGC STM1987, resulting in an increase in c-di-GMP upon exposure to L-arginine. Therefore, the use of a FRET-based biosensor can help define signals and pathways important to bacterial sensing.

RESULTS

Flow cytometry–based analysis identifies changes in free c-di-GMP concentrations in response to environmental nutrients

We have previously used a FRET-based c-di-GMP biosensor to characterize the amount of c-di-GMP in live bacteria using microscopy (*20*–*22*). However, microscopy is not amenable to high-throughput applications and requires that the bacterial sample is centrifuged, concentrated, and placed on an agar pad, which may result in changes to the amount of free c-di-GMP during preparation. Therefore, we developed methods to measure biosensor activity in *S.* Typhimurium strains by flow cytometry. This approach supports semi–high-throughput application, requires no manipulation of liquid live samples, and allows determination of relative free c-di-GMP in real time.

The c-di-GMP biosensor is based on the c-di-GMP–binding protein YcgR sandwiched between cyan fluorescent protein (CFP) and yellow fluorescent protein (YFP) fluorophores. When CFP is excited, some energy is transferred to the YFP fluorophore, by FRET, resulting in YFP emission. Upon binding to c-di-GMP, the ratio of YFP to CFP emission decreases; thus, low FRET values correspond to high amounts of c-di-GMP and vice versa. We first verified that flow cytometry accurately detected FRET intensity and discriminated between strains expressing a heterologous DGC or PDE. Indeed, compared to wild-type organisms, a strain expressing a DGC exhibited reduced FRET, whereas a strain expressing a PDE exhibited increased FRET (Fig. 1). These results indicate that flow cytometry can be used to determine FRET activity of the biosensor and the relative amounts of c-di-GMP in the bacterial population.

We hypothesized that environmental nutrients modulated the amount of c-di-GMP, because these would be encountered by *S.* Typhimurium during infection. Hence, we screened a library of 379 carbon, nitrogen, phosphate, and sulfur nutrient sources from phenotypic microarray plates (see Materials and Methods) for their ability to alter the concentration of c-di-GMP in *S.* Typhimurium. Background fluorescence of each compound

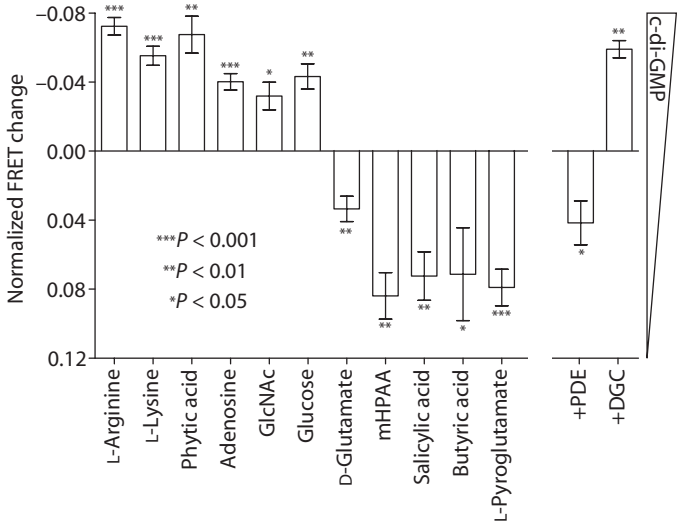

Fig. 1. Independent analysis of compounds identified in the nutrient screen results in alteration of c-di-GMP concentrations of *Salmonella* Typhimurium. Wild-type *S.* Typhimurium expressing the biosensor was incubated with each of the indicated compounds and a buffer-only control in six to seven independent experiments. Control strains of *S.* Typhimurium expressing a heterologous DGC or PDE were separately analyzed in three independent experiments. FRET was analyzed by flow cytometry, and the change in FRET/CFP ratios was analyzed for significance using paired *t* test comparing each compound to the buffer-only control. Values were then normalized to the buffer-only control and graphed to indicate the normalized change in FRET levels. Note that the *y* axis is plotted with negative values up, so that compounds that increased c-di-GMP concentration are indicated with upward bars and those that decreased c-di-GMP are indicated by downward bars. GlcNAc, *N*-acetyl-D-glucosamine; mHPAA, *m*-hydroxyphenylacetic acid. Compounds were used in the following concentrations: L-arginine (100 μM), L-lysine (2 mM), phytic acid (2 mM), adenosine (2 mM), GlcNAc (0.2 mM), glucose (1 mM), D-glutamate (0.5 mM), mHPAA (2 mM), salicylic acid (2 mM), butyric acid (12 mM), and L-pyroglutamate (8 mM).

was measured, and those that fluoresced in CFP or YFP wavelengths were removed from analysis (data S1, "Raw Screen Data"). We grew *S.* Typhimurium expressing the biosensor to early log phase and incubated the bacteria with each compound for 40 to 80 min before analysis by flow cytometry.

We sorted compounds on the basis of their effect on FRET levels and picked 11 compounds from this screen—chosen by their effect on FRET, biological relevance, and availability—to test in additional assays to verify the screen results (data S1). Bacteria were incubated with low millimolar concentrations of each compound, and all compounds reproduced statistically significant changes in FRET as predicted from the screen (Fig. 1). These results indicate that this screening method is a robust approach for identifying compounds that alter concentrations of c-di-GMP.

Identified compounds alter a subset of individual cells within the *S.* Typhimurium population, leading to a different distribution of cells with distinct amounts of c-di-GMP

Flow cytometry reports the FRET value of the population but is not sensitive enough to provide single-cell resolution. Therefore, we studied the effect of the identified compounds at a single-cell level by microscopy.

S. Typhimurium incubated in buffer exhibited a range of c-di-GMP concentrations centered on the biosensor binding affinity (Fig. 2A, middle panel), suggesting that *S.* Typhimurium populations have heterogeneous c-di-GMP concentrations in the conditions studied, a phenomenon that has been previously observed in other growth conditions (*20*).

Incubation with each compound resulted in a statistically significant shift in the distribution of c-di-GMP concentrations for single cells (Fig. 2B).

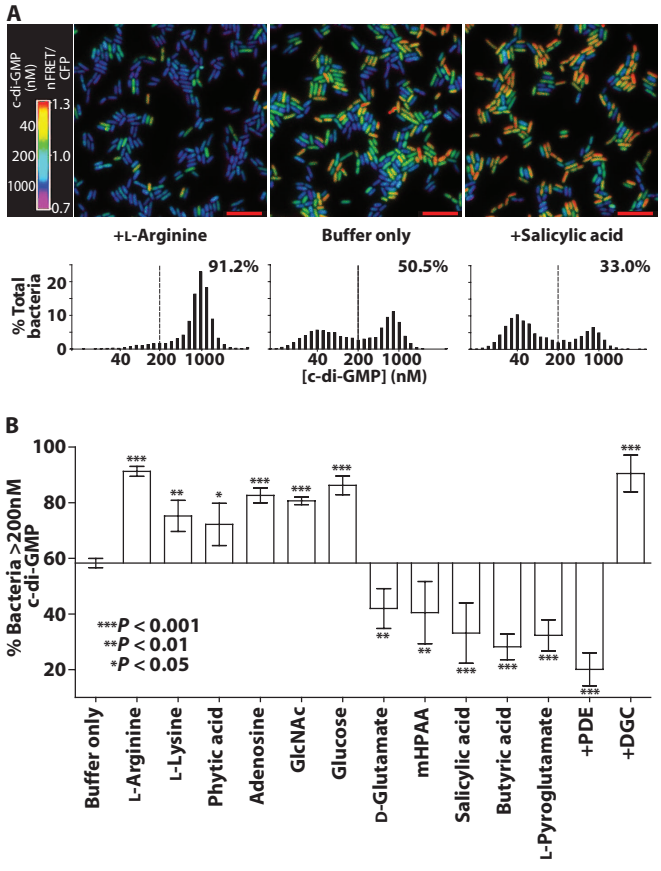

Fig. 2. Identified compounds modulate population heterogeneity of free c-di-GMP concentrations within individual cells. (A) *S.* Typhimurium wild-type cells expressing the biosensor were incubated with L-arginine, salicylic acid, or buffer only. Top: Representative fields displaying pseudocolored nFRET (normalized FRET values)/CFP ratios (intensity scale shows the calibration of nFRET/CFP to c-di-GMP in nM). Scale bars, 10 μm. Bottom: Quantification of c-di-GMP concentrations across the bacterial population using all fields captured in a representative experiment. The number on the top right is the percentage of the population displaying c-di-GMP concentrations above 200 nM. (B) Compounds identified in the screen shift the population heterogeneity of *S.* Typhimurium. Shown is the percentage of the population displaying c-di-GMP concentrations above 200 nM. Included are heterologous DGC- and PDE-expressing control strains. Data shown are the average of at least three independent experiments per compound. For each compound, between 1000 and 7000 bacteria were imaged per experiment. *P* values for unpaired *t* tests versus buffer only are shown. Compounds were used in the following concentrations: L-arginine (100 μM), L-lysine (2 mM), phytic acid (2 mM), adenosine (2 mM), GlcNAc (0.2 mM), glucose (1 mM), D-glutamate (0.5 mM), mHPAA (2 mM), salicylic acid (2 mM), butyric acid (12 mM), and L-pyroglutamate (8 mM).

Although addition of each compound induced a change in the distribution of c-di-GMP concentrations in the population, this did not result in uniformity—in all cases, populations still exhibited a wide distribution of c-di-GMP concentrations. Specifically, whereas under buffer-only conditions the population displayed 50% of the bacteria with c-di-GMP concentrations over the K_d (dissociation constant) of the YcgR biosensor (200 nM), this proportion changed to, at most, a 90% distribution of the population with greater than 200 nM upon addition of different compounds. Thus, intrinsic variability in *S.* Typhimurium populations results in variable c-di-GMP concentrations in the conditions tested. It also provides a buffering function for maintaining heterogeneity by preventing part of the population from reaching c-di-GMP effector–binding concentrations in response to a specific compound, thus maintaining responsiveness within the population.

Compounds that modulate intracellular free c-di-GMP concentrations also modulate cellulose synthesis

We hypothesized that changes in c-di-GMP concentrations may translate into changes in c-di-GMP–dependent bacterial behaviors. In *S.* Typhimurium, two c-di-GMP–binding effectors control cellulose synthesis, the cellulose synthesis modulator BcsE (*23*) and the cellulose synthase BcsA. We hypothesized that incubation with the identified compounds would influence cellulose synthesis. We found that, because flow cytometry enables the quantification of two light-scattering properties of particles, forward and side scatter, which should be altered in bacteria by changes in cellulose synthesis, we could monitor cellulose synthesis with this method (fig. S1). Cellulose synthesis may alter the light-scattering properties of the bacteria either directly by changing the size and complexity of a single bacterial particle or by causing bacterial agglutination, and light scattering has been used to study c-di-GMP–dependent agglutination of *Vibrio cholerae* (*24*). We therefore measured the percentage of the *S.* Typhimurium population showing high light scattering. Relative to wild type, expression of a heterologous DGC (PA1120 from *Pseudomonas aeruginosa*) increased the proportion of cells that exhibited high light scattering, whereas expression of a heterologous PDE (PA2133 from *P. aeruginosa*) decreased this proportion (Fig. 3A and fig. S1). We confirmed that these differences in light scattering required cellulose synthesis. Expression of either the DGC or the PDE in an *S.* Typhimurium mutant incapable of producing cellulose (Δ*bcsA*) resulted in similar light-scattering properties as the parent Δ*bcsA* strain (Fig. 3A).

As predicted, incubation of the wild-type bacteria, but not the Δ*bcsA* mutant, with the identified compounds resulted in changes in the proportion of cells with high light scattering, indicating changes in cellulose synthesis that were consistent with observed changes in c-di-GMP concentrations (Fig. 3B). The rapidity of the response was determined by measuring the proportion of cells with high light scattering in *S.* Typhimurium cultures incubated for increasing lengths of time with L-arginine or L-pyroglutamate. Changes in cellulose synthesis were statistically significant from buffer-only incubation after 5.5 min of incubation with L-arginine and 8.5 min of incubation with L-pyroglutamate (Fig. 3C). Thus, changes in c-di-GMP concentrations induced by incubation with the identified compounds were rapidly translated into changes in the c-di-GMP–responsive behavior of cellulose synthesis.

S. Typhimurium specifically senses L-arginine among the 20 common amino acids and responds by increasing c-di-GMP concentrations

We next characterized the effective concentrations of the various compounds. We determined the lowest concentration required to induce a significant change in the proportion of high light-scattering cells, indicating

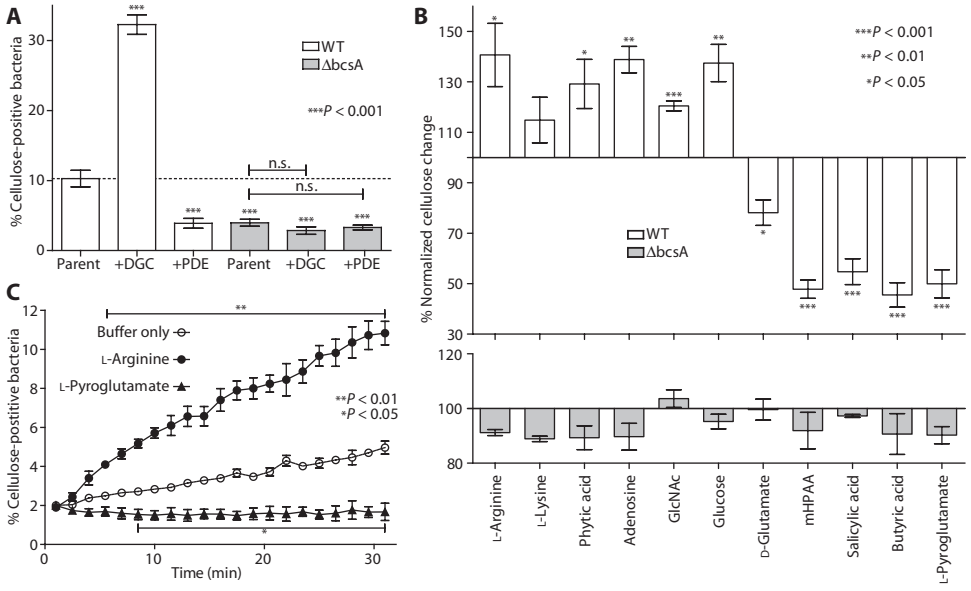

Fig. 3. Changes in c-di-GMP concentration rapidly modulates cellulose synthesis upon exposure to the identified compounds. (A) Alteration of c-di-GMP concentrations by expression of a heterologous DGC or PDE modulates light scattering and cellulose synthesis in a BcsA-dependent manner. Wild-type (WT) *S.* Typhimurium and Δ*bcsA* parent strains expressing either a DGC or a PDE were analyzed for cellulose synthesis by flow cytometry. Values are an average of three independent experiments. *P* values for paired *t* test versus the WT parent strain are shown, unless indicated by brackets. n.s., not significant. (B) *S.* Typhimurium WT or Δ*bcsA* bacteria incubated with the compounds identified in the screen were analyzed for cellulose synthesis using flow cytometry. Values were normalized to strains incubated with buffer only (100%) and are the average of three independent experiments. Data were analyzed by unpaired *t* test versus the buffer-only control. No Δ*bcsA* values were significantly different from the buffer-only control. (C) *S.* Typhimurium cells incubated with L-arginine or L-pyroglutamate rapidly alter cellulose synthesis. Bacteria were incubated with each compound and analyzed for changes in the population's light-scattering properties over time. Shown are the results of three independent experiments. Values were normalized to the initial time point (2.00%), and significance was determined by unpaired *t* test relative to the buffer-only sample at each time point. Brackets indicate the first time point in which all further time points are statistically significant. Compounds were used in the following concentrations: L-arginine (100 μM), L-lysine (2 mM), phytic acid (2 mM), adenosine (2 mM), GlcNAc (1 mM), glucose (1 mM), D-glutamate (1 mM), mHPAA (10 mM), salicylic acid (2 mM), butyric acid (12 mM), and L-pyroglutamate (8 mM).

a change in cellulose synthesis, and the concentration at which the maximal response was obtained (Fig. 4, A and B). The maximal response concentration is defined as the lowest concentration at which the greatest response in cellulose synthesis was observed.

Most compounds had threshold values in the micromolar range, indicating potential biological relevance (Table 1). L-Arginine was exceptional with a threshold value far lower than that of any other molecule assayed (0.128 μM), and L-arginine also achieved the highest maximal response at a very low concentration (0.64 μM). Using both changes in population distribution of c-di-GMP concentration measured by microscopy and cellulose synthesis measured by flow cytometry, we estimated an EC_{50} (median effective concentration) for L-arginine of 1.618 ± 0.615 μM and 0.205 ± 0.024 μM, respectively. These results indicate that *S.* Typhimurium generated a maximal c-di-GMP and cellulose synthesis response upon exposure to very low L-arginine concentrations.

In addition to L-arginine, we also identified five other L-amino acids that increased c-di-GMP: L-lysine, L-glutamate, L-aspartate, L-cysteine, and L-histidine (data S1). We determined that *S.* Typhimurium sensed L-lysine, which produced the second greatest response of the L-amino acids, at a 3000-fold higher concentration than L-arginine (Table 1). To determine whether L-arginine was specifically sensed at very low concentrations, we incubated *S.* Typhimurium with a mixture containing 100 μM of each of the 19 other L-amino acids plus variable L-arginine concentrations. Although

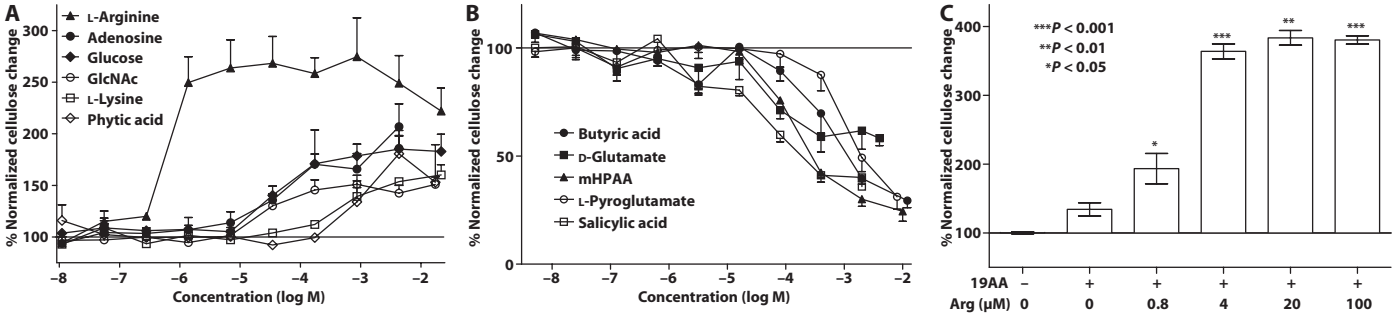

Fig. 4. L-arginine specifically stimulates c-di-GMP–dependent cellulose production at micromolar concentrations. (A) Dose-response analysis of the identified compounds that stimulate cellulose synthesis. (B) Dose-response analysis of the identified compounds that inhibit cellulose synthesis. Values in (A) and (B) were normalized to the buffer-only sample (100%) and are the average of three independent experiments. Values from these experiments were analyzed by unpaired *t* test to the buffer-only sample (see Table 1 for statistical significance information). (C) L-Arginine is specifically sensed among the 20 common amino acids at low concentrations. *S.* Typhimurium incubated with increasing concentrations of L-arginine with and without 100 μM of each of the other 19 L-amino acids (19AA) was tested for cellulose synthesis by flow cytometry. Data are the average of three independent experiments and were analyzed by paired *t* test for samples containing L-arginine and the 19 amino acid mix ($Arg^+/19AA^+$) to the control sample containing just the 19 amino acid mix ($Arg0/19AA^+$). Values were then normalized to the $Arg0/19AA^-$ sample (100%).

Table 1. Effective concentrations of identified compounds on cellulose synthesis.

Compound	CME identified as required for response	Threshold (μM; $P < 0.05$)*	Maximum response (μM)†	Extent of maximal response (%)‡
		Compounds that increased c-di-GMP		
L-Arginine	STM1987	0.128	0.64	260.6
N-acetyl-D-glucosamine	STM1987	16	80	147.5
Glucose	STM1987	16	80	179.4
Adenosine	STM2123	400	2,000	206.4
L-Lysine	STM1987	400	2,000	156.9
Phytic acid	STM1987	2,000	2,000	180.6
		Compounds that decreased c-di-GMP		
Salicylic acid	—	3.2	400	21.8
D-Glutamate	—	80	400	58.1
m-Hydroxyphenylacetic acid	—	80	10,000	22.5
Butyric acid	—	400	12,000	28.6
L-Pyroglutamate	STM2410	2,000	8,000	28.7

*Threshold is the lowest concentration of a compound that results in a statistically significant change in the percentage of high light-scattering bacteria in comparison to the buffer-only control. †Minimal concentration in which cellulose synthesis is at maximum. Note that in some cases in which a range of concentrations resulted in a maximal response (values are close to one another and are not significantly different from each other), only the lowest concentration is shown. ‡Maximum cellulose synthesis in response to each compound compared to the buffer-only control (100%).

incubation with the 19 other L-amino acids (and without L-arginine) resulted in a small increase in cellulose synthesis (Fig. 4C), addition of even just 0.8 μM L-arginine resulted in a statistically significant increase in cellulose synthesis above the 19 other amino acids. Thus, of the 20 commonly used L-amino acids, L-arginine was specifically sensed even when its concentration was 100-fold lower than that of the 19 other L-amino acids. This result suggests that L-arginine may be an important environmental signal sensed by *S.* Typhimurium, resulting in generation of c-di-GMP.

S. Typhimurium requires a specific DGC with a periplasmic domain to increase c-di-GMP concentrations in response to L-arginine in a translation-independent manner

Having identified several signals sensed by *S.* Typhimurium that resulted in a subsequent change in c-di-GMP concentration, we sought to characterize the pathways involved in the response to these compounds. After finding that changes in cellulose synthesis in response to L-arginine and L-pyroglutamate occurred rapidly (Fig. 3C), we tested the response to these compounds in the presence of the translation inhibitor chloramphenicol to determine whether regulation of c-di-GMP required new protein translation. We incubated *S.* Typhimurium expressing the biosensor with L-arginine, L-pyroglutamate, adenosine, or salicylic acid with and without chloramphenicol, measured the concentrations of c-di-GMP by microscopy, and plotted the proportion of cells with greater than 200 nM c-di-GMP (fig. S2). All four compounds induced a similar change in c-di-GMP concentrations irrespective of the presence of chloramphenicol, suggesting that all four compounds affect changes in c-di-GMP concentration through a post-translational mechanism.

Because protein translation was not required for the effect of the tested compounds, we hypothesized that altered activity of an *S.* Typhimurium c-di-GMP–modulating enzyme (CME) resulted in the changes to c-di-GMP concentrations. To identify the CME(s), we prepared a mutant library of all 17 annotated *S.* Typhimurium CMEs and used semi–high-throughput flow cytometry to rapidly screen the mutant library with the 11 tested compounds. Because of the robust response of *Salmonella* to L-arginine, we could clearly define primary and secondary CMEs in L-arginine sensing. When comparing the change in c-di-GMP concentrations upon addition of L-arginine, the 95% confidence interval (CI_{95}) for the mean was well past zero for 16 of the 17 mutants, showing that they still responded to L-arginine (Fig. 5A). Only deletion of STM1987, a putative DGC, resulted in a CI_{95} that includes zero, showing that this mutant was "blind" to the presence of L-arginine. The response of the Δ*stm1987* mutant was also significantly different from that of wild type. Thus, these results establish STM1987 as required for the L-arginine response and indicate it as the primary CME for L-arginine sensing. However, three other mutants—Δ*stm1827*, Δ*stm2215*, and Δ*stm4264*—all encoding predicted PDEs, also exhibited a statistically significant reduction in the response to L-arginine compared to that of wild type (Fig. 5A). These could be either experimental artifacts or secondary downstream contributing CMEs. Indeed, Δ*stm4264* and Δ*stm1827* had high background c-di-GMP concentrations when incubated in buffer only, potentially reducing biosensor sensitivity to signals that increase c-di-GMP (Fig. 5B).

In addition to L-arginine, we identified primary CMEs for six other compounds (fig. S3): STM1987 was also required to increase c-di-GMP in response to GlcNAc, glucose, phytic acid, and L-lysine, implying that it was a hub for nutrient sensing; STM2410, a putative PDE, was required to decrease c-di-GMP in response to L-pyroglutamate; and STM2123, a putative DGC, was required to increase c-di-GMP in response to adenosine. Because of data variation in the response to some compounds, we could not exclude potential requirement of some CMEs, making it possible that additional CMEs play a role in the response to GlcNAc, glucose, L-lysine, adenosine, and L-pyroglutamate. Furthermore, we could not identify a primary CME for the response to salicylic acid, D-glutamate, *m*-hydroxyphenylacetic acid, and butyric acid. This could be because these compounds act in a nonspecific manner on c-di-GMP concentrations, these compounds are sensed by multiple pathways that modulate the activity of multiple CMEs, or data variation did not allow identification of the CME.

We selected the STM1987-dependent L-arginine response for additional analysis because L-arginine exhibited the lowest effective concentration. As expected, in response to L-arginine, we did not detect any change in the proportion of cells showing high light scattering for the Δ*stm1987* strain, indicating that this strain did not synthesize cellulose in response to L-arginine (Fig. 5C). Complementation of Δ*stm1987* with a plasmid-encoded copy of wild-type *stm1987* ($p1987_{wt}$) restored both c-di-GMP modulation (Fig. 5D) and cellulose synthesis (Fig. 5C) in response to L-arginine. However, substituting a mutant version of STM1987 in which the canonical

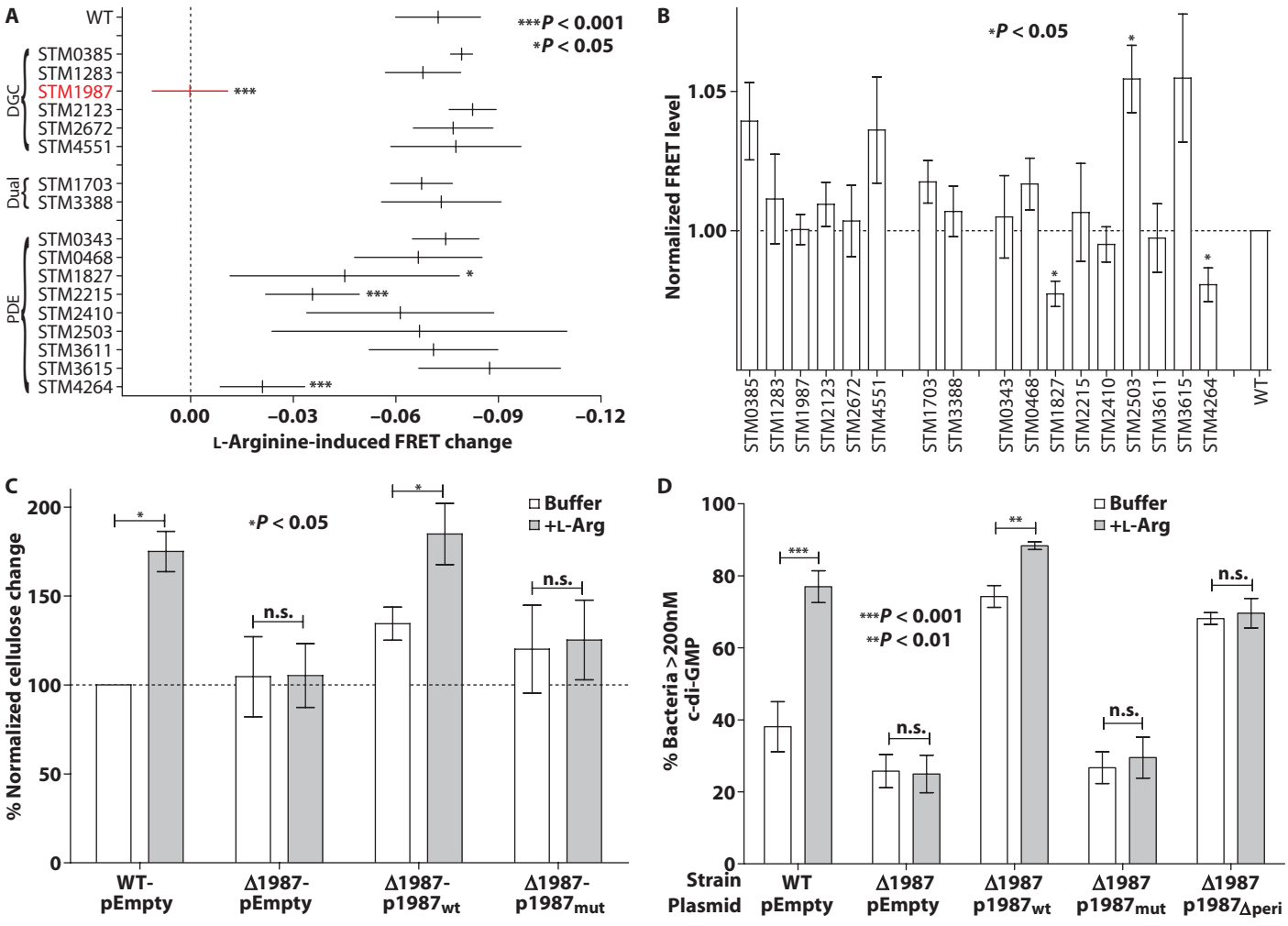

Fig. 5. The DGC STM1987 is required for the posttranslational L-arginine–induced increase in c-di-GMP. (**A**) Ability of the indicated mutant strains to alter c-di-GMP concentration in response to L-arginine. The nonresponsive Δ*stm1987* mutant is marked in red. WT and deletion mutants of each *S.* Typhimurium CME were incubated with or without L-arginine (10 μM). Strains are grouped upon the predicted activity of the protein based on the conservation of active-site residues in DGC and PDE domains, with "Dual" proteins containing both. Shown are changes in FRET values measured by flow cytometry for each strain in the presence of L-arginine normalized to the FRET values of the same strain in a buffer-only control (0.00). Graphed are the means (vertical lines, three to four independent experiments) and CI_{95} (horizontal lines) of the corresponding mutants compared to the buffer-only control for each mutant. The dashed line represents a value of 0, which indicates nonresponsiveness to L-arginine. Comparison to WT was calculated by unpaired *t* test of data normalized to the individual buffer-only control for each strain, and statistical significance is indicated. (**B**) Baseline concentrations of free c-di-GMP in the CME mutant strains. Data were analyzed for statistical significance by paired *t* test for each mutant versus the WT strain from three to four independent experiments. Shown are the average population FRET ratios of each mutant normalized to FRET levels from the WT strain (1.00). (**C**) *S.* Typhimurium WT or an Δ*stm1987* mutant (Δ1987) strain carrying an empty plasmid (pEmpty), a plasmid expressing WT STM1987 ($p1987_{wt}$), or a plasmid expressing a catalytically inactive mutant ($p1987_{mut}$) was measured for changes in cellulose production upon addition of either buffer alone or L-arginine (100 μM). Shown is the average relative cellulose synthesis determined by flow cytometry normalized to the WT buffer-only sample from three independent experiments. Statistical analysis was conducted by paired *t* test between L-arginine samples and the buffer-only control of each strain. Data were then normalized to the WT-pEmpty buffer-only control strain (100%) for presentation. (**D**) Changes in c-di-GMP concentrations in response to L-arginine (100 μM) in the indicated bacteria. $p1987_{\Delta peri}$ is the Δ1987 strain with a plasmid expressing STM1987 lacking the periplasmic Cache1 domain. Shown is the percentage of the population displaying c-di-GMP concentrations above 200 nM determined by microscopy from three to seven independent experiments. Statistical significance was calculated by paired *t* test between L-arginine samples and the buffer-only controls.

GGDEF active site was changed to GGAAF ($p1987_{mut}$) did not respond to L-arginine with a change in c-di-GMP concentration or in cellulose synthesis, indicating that the DGC activity of STM1987 is required for both responses.

In addition to the C-terminal GGDEF DGC domain, STM1987 also contains an N-terminal periplasmic Cache1 domain situated between two TM domains. We hypothesized that the periplasmic domain was required

for detecting L-arginine and regulation of c-di-GMP concentrations. Complementing with a mutant version of STM1987 containing both TMs but lacking its periplasmic domain ($p1987_{\Delta peri}$) resulted in a higher background concentration of c-di-GMP in the absence of L-arginine (Fig. 5D). This increase was similar to the background seen in the strain complemented with wild-type STM1987 ($p1987_{wt}$), implying that $STM1987_{\Delta peri}$ was enzymatically active. However, the strain expressing $STM1987_{\Delta peri}$, unlike the strain expressing $STM1987_{wt}$, did not respond to the presence of L-arginine by increasing c-di-GMP concentrations, indicating that the periplasmic portion of the protein was required for L-arginine sensing.

In addition to identifying a specific CME, the DGC STM1987, involved in the response to L-arginine, these results also demonstrate that combining the c-di-GMP biosensor with flow cytometry is an effective approach to rapidly screen a CME mutant library to identify specific enzymes required for sensing and generation of second messengers in response to specific cellular signals. Using these methods, we identified several CMEs potentially required for the response to a number of compounds. Furthermore, this approach is also useful in defining functional domains and exploring mechanisms of regulation, as represented by the identification that the response to L-arginine required the periplasmic Cache1 domain of STM1987 and occurred posttranslationally.

Increasing the concentration of c-di-GMP in response to L-arginine requires ArtI, a periplasmic putative L-arginine–binding protein

To further characterize the L-arginine–sensing pathway, we generated a number of deletion mutants in known L-arginine–sensing pathways and transporters. We found that a strain lacking ArgR, the central transcriptional regulator for L-arginine in the cytoplasm, was not required for cellulose synthase in the presence of L-arginine (Fig. 6A), which supports our conclusion that the mechanism by which L-arginine is sensed and stimulates an increase in c-di-GMP concentration occurs posttranslationally, because ArgR-mediated effects would be inhibited by chloramphenicol. In *P. aeruginosa*, the chemotaxis machinery regulates c-di-GMP concentrations (*22*); therefore, we created a Δ*cheA* strain, which is defective in chemotaxis. However, this strain responded to L-arginine with an increase in c-di-GMP, suggesting that sensing was independent of chemotaxis (fig. S4). We also created strains mutated in each of the three annotated L-arginine transport systems of *S.* Typhimurium (*25*–*27*). Whereas the HisJQMP and AdiC transport systems were not required for L-arginine sensing, a strain in which four genes of the Art transport system were deleted (*artPIQM*) did not respond to the presence of L-arginine by increasing either cellulose synthesis or c-di-GMP concentrations (Fig. 6, B and C).

The Art transport system compromises five genes (*25*) encoding the permeases ArtQ and ArtM, the cytoplasmic ATPase (adenosine triphosphatase) ArtP, and the PBP ArtI in one operon, and the PBP ArtJ, which is transcribed from a second promoter. Both ArtI and ArtJ are annotated as L-arginine PBPs, and most residues important for L-arginine binding are conserved (fig. S5) (*28*). A strain lacking only the permeases ArtQ and ArtM and a strain lacking only ArtJ responded to L-arginine, implying a role for the PBP ArtI in L-arginine sensing (Fig. 6A). To verify a requirement for ArtI in the c-di-GMP response to L-arginine, we complemented the Δ*artPIQM* strain in trans with a plasmid expressing only *artI*. Indeed, this strain increased cellulose synthesis and c-di-GMP concentration in response to L-arginine (Fig. 6, B and C). Strains mutated in other annotated L-arginine PBPs (*28*)—ArtJ, ArgT, or STM4351—responded to L-arginine (Fig. 6A), implying a specific role for ArtI. Together with the requirement for the periplasmic domain of STM1987, these data suggested that signaling of L-arginine occurs within the periplasm of *S.* Typhimurium and requires both the periplasmic putative L-arginine–binding protein ArtI and the DGC STM1987.

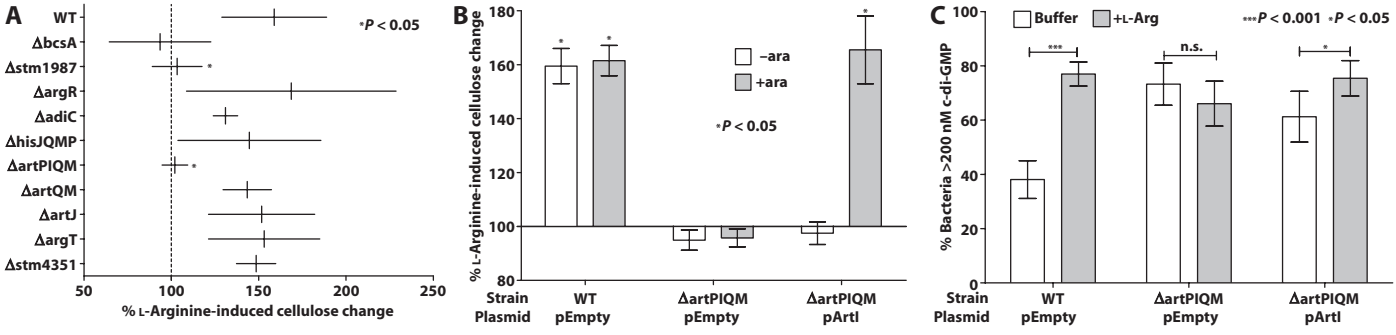

Fig. 6. The L-arginine–induced increase in c-di-GMP concentrations requires the periplasmic putative L-arginine–binding protein ArtI. (**A**) Deletion mutants of an L-arginine–responsive transcriptional regulator (ArgR), L-arginine transport systems (AdiC, HisJQMP, and ArtPIQM), and L-arginine transport components (ArtQM, ArtJ, ArgT, and STM4351) were tested with WT *S.* Typhimurium (WT), Δ*bcsA*, and Δ*stm1987* mutants for cellulose synthesis in response to L-arginine (100 μM). Changes in cellulose synthesis were measured by flow cytometry in three independent experiments. Graphed are the means (vertical lines) and CI_{95} (horizontal lines) of the corresponding mutants compared to the buffer-only control for each mutant. Data were normalized to the buffer-only control for each strain (100%). Statistical significance between strains was determined by paired *t* test versus the WT strain. (**B** and **C**) ArtI is sufficient to restore L-arginine–dependent c-di-GMP production and cellulose export to the Δ*artPIQM* mutant. Complementation of the Δ*artPIQM* mutant was conducted by an arabinose-inducible promoter. (B) Normalized change in cellulose synthesis upon addition of L-arginine (100 μM) versus buffer alone with and without induction of the plasmid expressing ArtI (pArtI, +/−ara) from three independent experiments. Data were analyzed before normalization for statistical significance by paired *t* test for each sample with L-arginine versus that sample in a buffer-only control. Data were then normalized to the buffer-only control for each condition (100%) for presentation. (C) Strains grown in the presence of arabinose and after addition of either buffer alone or L-arginine (100 μM) measured using microscopy to quantify the percentage of cells showing c-di-GMP concentrations above 200 nM. Statistical significance was calculated by paired *t* test for each strain and condition between L-arginine and buffer-only samples from three independent experiments.

DISCUSSION

Bacteria must sense changing environments to properly regulate responses that ensure their survival. Multiple sensing pathways modulate c-di-GMP concentrations to regulate bacterial behaviors associated with motility and biofilm formation. *S.* Typhimurium encodes 17 putative CMEs, many with predicted sensing domains, yet little is known of what activates them (*6*, *18*, *29*). Here, we designed a method to study relative c-di-GMP concentrations in semi–high throughput using a c-di-GMP biosensor in conjunction with flow cytometry. Using this method, we conducted a screen for c-di-GMP–modulating compounds and identified multiple compounds that alter c-di-GMP concentrations of *S.* Typhimurium. Eleven compounds were further characterized and were found to change the distribution of c-di-GMP concentrations in the bacterial population. All 11 also affected cellulose synthesis in a manner expected from their modulation of c-di-GMP concentrations, with those that increased c-di-GMP stimulating cellulose synthesis and those that decreased c-di-GMP reducing cellulose synthesis, and dependent on the c-di-GMP–activated cellulose synthase BcsA. We used the same semi–high-throughput approach to probe the associated signaling pathways by screening a library of mutant strains, which resulted in identification of specific enzymes required for the response to several specific compounds that function as bacterial signals and change second-messenger concentrations. These results illustrate the use of this method to quickly and accurately identify signals sensed by bacteria and, in particular, to begin to isolate the signaling pathways involved in the c-di-GMP response.

The use of the biosensor in conjunction with flow cytometry has advantages over other traditional methods of c-di-GMP quantification: this method supports semi–high-throughput application, requires no manipulation of liquid live samples, and enables determination of relative free c-di-GMP concentrations in real time. Measuring rapid responses and directly quantifying second-messenger concentrations are more likely to identify signals that have direct immediate effects, rather than result from cumulative changes over an extended incubation period, such as those involving gene expression and protein production. One limitation of the screen that we performed here is that the YcgR-based biosensor is limited in use to environmental conditions that result in c-di-GMP concentrations within the sensitivity range of YcgR binding affinity (40 to 1000 nM). However, this limitation can be overcome by the use of biosensors with YcgR mutations or alternative c-di-GMP–binding domain-based biosensors that have different affinities for c-di-GMP (*21*). Using conditions in which normal c-di-GMP concentrations are within the sensitivity range of the biosensor also allowed the same screen to identify compounds that both increased or decreased c-di-GMP concentrations. Different biosensors could be used in the future to expand the range of media conditions and signals beyond what was screened in this work.

Characterization of selected compounds revealed that several had threshold values in the micromolar range, indicating potential biological relevance (Table 1). Nevertheless, even those compounds with higher threshold values may be relevant; for instance, adenosine, which was found to maximally respond at a concentration of 2 mM, is estimated to reach concentrations of 5 mM in the lumen of the intestine, an important environment for *S.* Typhimurium (*30*). Additionally, *S.* Typhimurium responded to four compounds (glucose, GlcNAc, salicylic acid, and L-arginine) at concentrations below 20 μM. Whereas glucose is potentially sensed as an energy source, GlcNAc is a component of both peptidoglycan and the eukaryotic extracellular matrix, suggesting possible sensing of bacterial or host compounds, or both, within the intestinal tract where both extracellular matrix and the microbiome will create a robust signal relevant to *S.* Typhimurium (*31*).

One potential application of our method is in searching for compounds that could reduce c-di-GMP to inhibit biofilm formation in clinical settings. Of the compounds tested, only low concentrations of salicylic acid acutely reduced c-di-GMP. Salicylic acid inhibits *S.* Typhimurium biofilm formation, and identification of salicylic acid as a reducer of c-di-GMP concentrations indicates that c-di-GMP is likely the mechanism of biofilm inhibition (*32*). It is interesting to speculate that salicylic acid, a plant hormone, may be secreted upon bacterial infection of plant tissues to inhibit bacterial colonization and biofilm formation through inhibition of c-di-GMP synthesis (*33*). The assays developed here may be useful to screen for compounds that can inhibit increases in bacterial c-di-GMP and subsequent biofilm formation in vivo.

Among the compounds identified, L-arginine both induced the highest level of response and reached maximal response at a very low concentration. Additionally, L-arginine induced an increase in c-di-GMP even when incubated with the other 19 commonly used amino acids, suggesting that the response is specific to L-arginine and is not a reaction to the addition of nitrogen or amino acids. *S.* Typhimurium stimulates expression of host L-arginine importers along the *Salmonella*-containing vacuole during infection, implying that L-arginine could be an important signal sensed by *S.* Typhimurium and may indicate the intracellular environment (*34*). Furthermore, *S.* Typhimurium mutants lacking L-arginine transporters are attenuated during infection of inbred mice models, implying that L-arginine is important for virulence (*35*).

We identified the DGC STM1987 as required for the response to L-arginine, L-lysine, phytic acid, GlcNAc, and glucose, indicating that STM1987 may be a hub for integration of nutrient sensing. A mutant in *stm1987* is impaired in a mouse model of infection (*36*), indicating that sensing through STM1987 may be important for virulence. Furthermore, L-arginine rapidly stimulated c-di-GMP–dependent cellulose synthesis by *S.* Typhimurium. Cellulose formation in *S.* Typhimurium increases resistance to desiccation and sodium hypochlorite, enhances long-term survival, and enhances attachment to plants (*37*–*40*). Recently, cellulose was shown to be synthesized by intracellular *S.* Typhimurium (*41*), suggesting a role for cellulose in the *S.* Typhimurium vacuole and a potential site of L-arginine sensing. In addition to BcsA, *S.* Typhimurium encodes multiple other putative c-di-GMP effectors, suggesting further potential mechanisms of the role of c-di-GMP during infection (*23*).

STM1987, the DGC that we found was required for sensing L-arginine, L-lysine, phytic acid, glucose, and GlcNAc, contains a periplasmic Cache1 domain that was required for L-arginine–sensing (Fig. 5D). Cache domains are also found in MCPs and HKs, and multiple examples exist of MCPs, HKs, and CMEs regulated by the binding of PBPs that act as the actual environment-sensing proteins, although to date none of these examples include a protein with a Cache domain (*10*–*15*). Indeed, we showed that ArtI, an annotated L-arginine–binding PBP, is required for L-arginine sensing. Although we cannot exclude a cytoplasmic pathway for sensing of L-arginine in which ArtI is required to transport L-arginine into the cytoplasm through an alternative pathway outside of the ArtPIQM ABC transporter, we favor a periplasmic pathway of sensing (Fig. 7). First, we found that L-arginine sensing occurred in the absence of the rest of the Art transporter components, so transport would have to occur through a different ABC transporter family. Because neither a single deletion of the HisJQMP nor AdiC transport systems inhibited sensing, a putative ArtI-dependent L-arginine transport activity would have to be promiscuous and use more than one transport system, which is less likely. Second, we showed that the periplasmic domain of STM1987 is required for L-arginine sensing. Third, because the cytoplasmic portion of STM1987 contains only the GGDEF DGC domain, a cytoplasmic route of L-arginine sensing would require the activation of the DGC domain by L-arginine

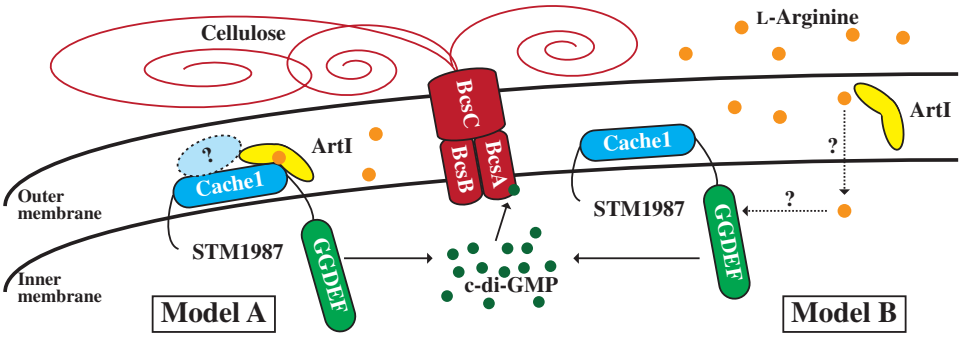

Fig. 7. Model for *S.* Typhimurium c-di-GMP regulation in response to L-arginine. Two possible pathways of L-arginine sensing can explain the chain of events leading to STM1987 activation by L-arginine, a periplasmic-sensing pathway (Model A) or a cytoplasmic-sensing pathway (Model B). In Model A, ArtI and STM1987 interact through the Cache1 domain of STM1987, either directly or indirectly through a protein complex. L-arginine binding to ArtI promotes the DGC activity of STM1987 mediated by the GGDEF domain, increasing c-di-GMP synthesis. In Model B, ArtI is required for sensing because of a putative L-arginine transport function independent of the cotranscribed ABC transporter apparatus. Once in the cytosol, L-arginine activates STM1987 by an unknown mechanism. Although we assayed the effect of changes in c-di-GMP on cellulose synthesis, this pathway may regulate other c-di-GMP effectors in this and other growth conditions.

or a derivative, which is a mechanism that has not yet been identified for any DGC. Fourth, we found that sensing was independent of the known L-arginine–binding cytoplasmic transcriptional regulator, ArgR, which is also consistent with L-arginine responsiveness in the presence of chloramphenicol, suggesting a posttranslational regulatory mechanism. Last, multiple characterized pathways include MCPs, HKs, and CMEs that contain coupled periplasmic domains, like STM1987 does, and bind PBPs for sensing (*10*–*15*). Together, these results suggest that ArtI, upon binding L-arginine, stimulates the DGC activity of STM1987 through the periplasmic Cache1 domain, either directly or as part of a protein complex (Fig. 7).

Sensing of changes in the environment is critical for the survival and adaptation of bacteria. Indeed, just the sheer number of proteins with signal transduction domains implies the existence of multiple uncharacterized signals that are sensed by bacteria. Because bacteria occupy diverse environments, different bacteria are expected to sense very different signals. Signal transduction pathways using c-di-GMP are ubiquitous in both Gram-negative and Gram-positive bacteria. The use of a real-time biosensor should have broad use for characterizing signals and sensing pathways in many different bacteria. Understanding the diverse signals that promote different behaviors in bacteria should provide insight into the life-style of these organisms and enable the identification of compounds that alter c-di-GMP concentrations and bacterial signaling. Further, these compounds could represent new therapeutic options for antimicrobial therapy in which bacterial biofilm formation and inducible antimicrobial resistance are inhibited through alteration of specific signal transduction pathways and their effector enzymes.

MATERIALS AND METHODS

Strains, plasmids, and primers

A list of strains and plasmids can be found in table S1, and a list of primers used for cloning can be found in table S2. All mutant strains were generated from *S.* Typhimurium SL14028 with the method described by Datsenko and Wanner (*42*). Mutations were then transduced by P22 phage transduction into wild-type or Δ*bcsA* backgrounds (*43*). Generation of STM1987 expression vectors was accomplished by cloning a polymerase chain reaction (PCR) product of STM1987 from the SL14028 genome using the primers indicated into the pCR2.1-Topo vector (Invitrogen). Site-directed mutagenesis was conducted on the pTopo-1987_{wt} vector to generate the 1987_{mut} version. Deletion of the periplasmic domain of STM1987 ($1987_{\Delta peri}$) was achieved by inverse PCR of the pTopo-$STM1987_{wt}$ vector using primers listed in table S2, followed by subsequent restriction digestion and ligation. All alleles were cloned into the pBAD24 vector using restriction enzyme sites found in the pBAD cloning primers.

Bacterial growth conditions

Bacteria were grown for about 17 hours at 30°C, shaking in 2 ml of a minimal defined medium [21 mM K_2HPO_4, 11 mM KH_2PO_4, 3.8 mM $(NH_4)_2SO_4$, ~3.8 mM KOH, 25 mM glycerol, 1 mM $MgCl_2$, 10 μM $FeCl_3$, 1 mM NaCl, 1× MEM amino acid solution (Gibco, 11130), 1× MEM nonessential amino acid solution (Gibco, 11140), pH 7.4]. Cultures were then diluted 1:100 and grown for an additional 2 hours at 30°C, shaking in 5 ml of the same medium lacking amino acid solutions. IPTG (isopropyl-β-D-thiogalactopyranoside) (500 μM for 17 hours overnight and 100 μM during 2-hour growth), L-arabinose (0.02%), gentamicin (15 μg/ml), and carbenicillin (50 μg/ml) were added as needed. Screen compounds were diluted in water and added in a volume of 1:5 of the bacteria. Bacteria were incubated with compounds for 30 to 40 min at 30°C standing before analysis by either flow cytometry or microscopy. For chloramphenicol experiments, chloramphenicol (34 μg/ml) was added after 2 hours of growth for 30 min before addition and during incubation with the various compounds. For the time course experiment, a 96-well plate was prepared with compounds, bacteria from the same culture were added, and analysis by flow cytometry was started immediately. Thus, each time point originates from a different well, and time values were determined on the basis of time required for analysis.

Flow cytometry and analysis

A BD LSR II with a high-throughput sampler was used for flow cytometry. Excitation of 488 nm was used for side scatter. FRET was quantified using 405-nm excitation and 540/40-nm or 525/50-nm filters. CFP was quantified using 405-nm excitation and 470/20-nm or 450/50-nm filters. YFP was quantified using 488-nm excitation and a 530/30-nm filter. YFP measurements were used in both flow and microscopy to ensure that expression of the biosensor was equal among samples. Data were analyzed by FlowJo v10, Microsoft Excel, and MatLab. Values reported are the averaged ratios of FRET over CFP for each particle.

Light scatter of bacterial particles was measured by flow cytometry to determine cellulose-positive bacteria. After data acquisition, a gate was set identifying the main population (~85% of particles) in a buffer-only or Δ*bcsA* control (fig. S1). The cellulose-positive gate was determined as those particles that exhibited higher levels of light scatter, excluding the main population. This gate was then used for all samples in the same 96-well plate to determine relative percentage of cellulose-positive bacteria.

Screen for compounds modulating c-di-GMP concentration

Phenotypic microarray plates 1 to 4 (PM1-4, Biolog) were used for the screen. Compounds were diluted in 50 µl of water, and 20 µl was transferred into a 96-well plate. SL14028 Δ*bcsA*/pMMBgm-Biosensor bacteria (80 µl) grown as described above were added to each well, and the plate was incubated standing at 30°C for 30 min before analysis by flow cytometer. Plates were analyzed in groups of 48 compounds, allowing for a blank well between each sample in a 96-well plate to prevent contamination of samples. Each phenotypic microarray plate was separated vertically (wells A1-H6 on one plate, wells A7-H12 on the other).

Each plate of the screen was repeated in three to five independent experiments. Background emission of the compounds at 470 and 535 nm in response to excitation at 430 nm was measured before analysis using an EnVision multilabel plate reader (PerkinElmer), and compounds showing fluorescence values greater than 15% above the plate average were excluded from analysis (data S1). This resulted in the exclusion of 23 compounds from analysis (6.0%). To control for the effect of general nutrient availability on c-di-GMP concentration, we compared the effect of a single compound on the average effect of a group of similar compounds. Because the phenotype microarray plates are grouped according to the nutrient type they supply (carbon, nitrogen, phosphate, and sulfur), we used the mean value of each set of 48 compounds that a particular compound was tested with as the control reference. Statistical significance for each compound versus the mean for the specific 48 compound set using the raw FRET/CFP ratio data was calculated using a paired *t* test (data S1, "Raw Screen Data"). To allow comparison of the extent of change of samples analyzed on different dates and different 96-well plates, each sample was normalized to the mean of the specific 48-compound set (set at 0.00), described as the change in FRET/CFP ratios versus the mean (ΔFRET). These values were averaged and are reported in data S1, which also includes the raw data and calculated mean values.

Microscopy and analysis

Microscopy and analysis of microscopy data were done essentially as described previously (*22*) with the exception that no binning of microscopic images was performed during acquisition. Bacteria with measurements outside the linear range of detection were excluded from analysis. FRET values were normalized (nFRET) to account for overlapping emission of CFP wavelengths into the YFP filter used for FRET. The concentration of c-di-GMP for a given bacterium was calculated from the nFRET/CFP ratio and the K_d of the biosensor at 25°C, as samples were placed onto 25°C agar pads and measured at 25°C (*20*).

SUPPLEMENTARY MATERIALS

www.sciencesignaling.org/cgi/content/full/8/380/ra57/DC1

Fig. S1. Gating by light scattering in wild-type bacteria and Δ*bcsA*.
Fig. S2. Identification of c-di-GMP CMEs required for response to identified compounds.
Fig. S3. Alteration of c-di-GMP levels in the presence of translation inhibitor chloramphenicol.
Fig. S4. CheA is not required for the increase in c-di-GMP in response to L-arginine.
Fig. S5. Alignment of ArtI protein with *S.* Typhimurium predicted L-arginine–binding proteins identifies conservation of residues required for L-arginine binding.
Table S1. Strains and plasmids used in this study.
Table S2. Oligonucleotides used in this study.
Data S1. Nutrient screen data.

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