

# Engineering of *Bacillus subtilis* Strains To Allow Rapid Characterization of Heterologous Diguanylate Cyclases and Phosphodiesterases

Xiaohui Gao,<sup>a,c</sup> Xiao Dong,<sup>a</sup> Sundharraman Subramanian,<sup>a,c</sup> Paige M. Matthews,<sup>a</sup> Caleb A. Cooper,<sup>a</sup> Daniel B. Kearns,<sup>b</sup> Charles E. Dann III<sup>a,c</sup>

Department of Chemistry,<sup>a</sup> Department of Biology,<sup>b</sup> and Biochemistry Graduate Program,<sup>c</sup> Indiana University, Bloomington, Indiana, USA

Microbial processes, including biofilm formation, motility, and virulence, are often regulated by changes in the available concentration of cyclic dimeric guanosine monophosphate (c-di-GMP). Generally, high c-di-GMP concentrations are correlated with decreased motility and increased biofilm formation and low c-di-GMP concentrations are correlated with an increase in motility and activation of virulence pathways. The study of c-di-GMP is complicated, however, by the fact that organisms often encode dozens of redundant enzymes that synthesize and hydrolyze c-di-GMP, diguanylate cyclases (DGCs), and c-di-GMP phosphodiesterases (PDEs); thus, determining the contribution of any one particular enzyme is challenging. In an effort to develop a facile system to study c-di-GMP metabolic enzymes, we have engineered a suite of *Bacillus subtilis* strains to assess the effect of individual heterologously expressed proteins on c-di-GMP levels. As a proof of principle, we characterized all 37 known genes encoding predicted DGCs and PDEs in *Clostridium difficile* using parallel readouts of swarming motility and fluorescence from green fluorescent protein (GFP) expressed under the control of a c-di-GMP-controlled riboswitch. We found that 27 of the 37 putative *C. difficile* 630 c-di-GMP metabolic enzymes had either active cyclase or phosphodiesterase activity, with agreement between our motility phenotypes and fluorescence-based c-di-GMP reporter. Finally, we show that there appears to be a threshold level of c-di-GMP needed to inhibit motility in *Bacillus subtilis*.

Bis-(3'-5')-cyclic dimeric GMP (c-di-GMP) is a ubiquitous secondary messenger that regulates bacterial processes, including biofilm formation, motility, and virulence (1–7). c-di-GMP is synthesized by diguanylate cyclases (DGCs) and hydrolyzed by c-di-GMP phosphodiesterases (PDEs) with conserved GGDEF domains and EAL or HD domains, respectively (8–18). The presence of c-di-GMP is sensed by many distinct receptor classes, including, but likely not limited to, PilZ domains, degenerate EAL domains, degenerate GGDEF domains, transcription factors, and riboswitches (19–40). Although most c-di-GMP signaling factors share consensus motifs that make them identifiable on the basis of sequence alone, characterization of c-di-GMP signaling in organisms with a large number of putative signaling factors remains a challenge.

Several microbial hosts, in a subset that includes *Escherichia coli*, *Pseudomonas aeruginosa*, *Pectobacterium atrosepticum*, *Vibrio cholerae*, and *Clostridium difficile*, have been utilized to screen for endogenous or heterologous active DGCs and PDEs (41–47). Depending on the host, the activity of putative enzymes can be assessed by analysis using a combination of Congo red staining (41, 42, 48), aggregation (42, 46, 48), motility (42, 44–47), biofilm formation (42, 45), and mass spectrometry (47, 49) assays. Many of these hosts, however, are pathogenic, contain complex endogenous c-di-GMP signaling components, or are difficult to genetically manipulate (42, 43, 45, 50). Another system in *Bacillus subtilis* offers many advantages, as *B. subtilis* is harmless and easy to grow and has facile genetic system (43, 50). Furthermore, *B. subtilis* contains a concise c-di-GMP signaling pathway comprised of three active DGCs (DgcK, DgcP, and DgcW), one active PDE (PdeH), and a single c-di-GMP receptor (DgrA), and strains lacking any combination of the aforementioned proteins have recently been reported (43). Finally, on the basis of current data, an in-

creased c-di-GMP level has a single clearly characterized biological consequence in *B. subtilis*, namely, inhibition of swarming motility.

Given the need for a reliable, nonpathogenic host to study c-di-GMP signaling components, engineered *B. subtilis* strains with elevated or absent c-di-GMP have been developed to examine the activity of putative PDEs or DGCs on the basis of a robust swarming motility phenotype (43). Additionally, we expected that a direct sensor for c-di-GMP might provide advantages over all current assays that rely on biological phenotypes. Thus, in this work we developed a fluorescence reporter on the basis of a designed, chimeric c-di-GMP riboswitch. Using two distinct output systems, swarming motility and single-cell fluorescence analysis, we analyzed 37 putative enzymes from *C. difficile* 630 for production or depletion of c-di-GMP (Fig. 1). As many of these *Clostridium* genes were examined previously for activity using the Gram-negative *V. cholerae* as a host (45), these targets serve to directly compare and assess the potential of Gram-positive *B. subtilis* as a general heterologous host to study c-di-GMP signaling.

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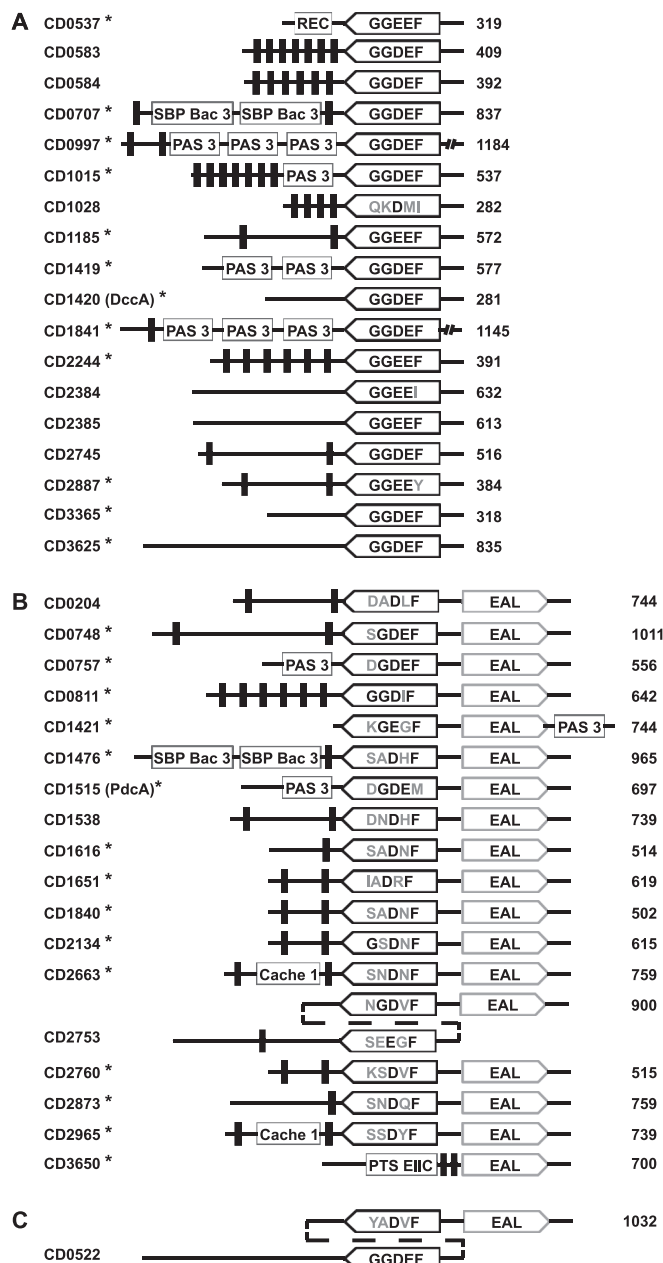
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Address correspondence to Charles E. Dann III, cedann@indiana.edu.

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**FIG 1** Domain architectures of the GGDEF and EAL proteins encoded by *C. difficile*. Proteins tested for DGC (A), PDE (B), and dual DGC and EAL activities (C) are shown. Asterisks mark proteins deemed to have DGC (A) or PDE (B) activity in this work. Black boxes represent predicted transmembrane regions. REC, receiver domain found in two-component signaling systems; SBP bac, domain found in bacterial extracellular solute-binding proteins; PAS, a sensory domain of the PER/ARNT/SIM family known to respond to oxygen, redox potential, and light in other systems; Cache 1, calcium channels and chemotaxis receptor family 1; PTS EIIC, phosphotransferase system EIIC. Proteins are not drawn to scale.

## MATERIALS AND METHODS

**Construction of heterologous expression strains.** To generate inducible translational fusion constructs for genes encoding putative diguanylate cyclases from *C. difficile* 630, our previously engineered strain, NPS236 ( $\Delta$ GGDEF *pdeH::kan amyE::Pc-dgrA*), served as the parent for the production of 17 constructs (pXG106 to pXG122). All GGDEF domain proteins

were amplified from *C. difficile* 630 genomic DNA (ATCC BAA-1382D-5) using primers GXH544 and GXH579. Amplicons were cloned into pXG101—which carries a gene conferring resistance to erythromycin and lincomycin (macrolide, lincosamide, and streptogramin [MLS] resistance), the *P<sub>hysp</sub>*-inducible promoter, and the *B. subtilis* *dgcP* leader sequence (nucleotides –60 to +3 relative to translational start site) flanked by segments of the *thrC* gene—for homologous recombination via isothermal assembly or standard ligation techniques (43, 51, 52). The homologous recombination into the *thrC* locus was confirmed by selection on minimal-medium plates lacking threonine.

To generate inducible translational fusion constructs for genes encoding putative c-di-GMP phosphodiesterases from *C. difficile* 630, our previously engineered strain, NPS235 (*pdeH::kan amyE::Pc-dgrA*), was used to create 19 constructs (pSS820 to pSS838). A total of 19 EAL domain protein gene cassettes were amplified from *C. difficile* 630 genomic DNAs using primers SS131 to SS257. Amplicons were cloned into pXG101 via isothermal assembly or standard ligation techniques (43, 51, 52). Constructs were confirmed by sequencing and transformed into a competent *B. subtilis* strain (DS2569) to generate phage lysates for transduction (53).

**Construction of c-di-GMP riboswitch reporter strains.** To construct a c-di-GMP-responsive biosensor, a chimeric riboswitch was engineered upstream of the coding sequence for green fluorescent protein (GFP) (54). Specifically, the biosensor was designed with nucleotides –564 to –86 of *B. cereus* *bc\_4140* (strain ATCC 14579)—containing an M-box riboswitch promoter, aptamer, transcriptional terminator, and flanking sequences—as a scaffold (39, 55). The M-box aptamer, nucleotides –469 to –321, was replaced with the aptamer sequence from a c-di-GMP-responsive riboswitch (GEMM motif), nucleotides –224 to –146, of *B. cereus* *bce\_0489* (strain ATCC 10987). To match the intrinsic terminator from the M-box expression platform to the P1 stem of the GEMM aptamer, seven mutations were made to the terminator to maintain terminator integrity while introducing mutually exclusive base pairing with a portion of the P1 stem of the GEMM aptamer to form an antiterminator. To facilitate cloning, the chimeric riboswitch was flanked by EcoRI and BglII restriction sites. Additionally, a G-to-A mutation was made in the M-box scaffold to ablate a native EcoRI restriction site. The entire nucleotide sequence for the chimeric c-di-GMP GFP reporter is included in Fig. S1 in the supplemental material.

The designed chimeric riboswitch was amplified from primers ID363 to ID376 and inserted into the EcoRI and BglII sites of pAM001, a vector containing GFP and a spectinomycin resistance cassette flanked by sequences from *B. subtilis* *amyE*, using isothermal assembly (51, 52, 56). The pAM001 plasmid was generated for this work via insertion of annealed primers AM005 and AM006 into pMF35 (54) linearized at EcoRI and HindIII sites to introduce a multiple-cloning site with NheI, SpeI, and SphI restriction sites. The resulting plasmid, pID024, was confirmed by sequencing and transformed into a competent *B. subtilis* strain (PY79) to generate phage lysates for subsequent transduction into *B. subtilis* strains DK391 and DK392 using SPP1 phage transduction, generating strains NPS400 and NPS401, respectively. Homologous recombination of the riboswitch reporter into the *amyE* locus was confirmed on starch plates (LB broth fortified with 1.5% agar and 1% starch) stained with an iodine solution (1% [wt/vol] iodine, 2% [wt/vol] potassium iodide). All *C. difficile* 630 GGDEF domain protein gene cassettes were introduced into the *thrC* locus of NPS401 using phage lysates from our strains used for swarming motility assays (NPS254, NPS287 to NPS303, and NPS342) to generate riboswitch reporter strains NPS402 to NPS420. All the *C. difficile* 630 EAL domain protein gene cassettes were similarly introduced into NPS400 using the phage lysates from strains NPS519 to NPS537 to generate riboswitch reporter strains NPS421 to NPS439.

**SPP1 phage transduction (53).** Stationary-phase cultures (200  $\mu$ l) grown in TY broth (LB broth supplemented with 10 mM  $MgSO_4$  and 100  $\mu$ M  $MnSO_4$  after autoclaving) were added to serial dilutions of SPP1 phage stock and statically incubated for 15 min at 37°C. To each mixture, 3 ml TYSA (molten TY supplemented with 0.5% agar) was added, and the

mixture was poured atop fresh TY plates and incubated at room temperature overnight. Top agar from plates that contained nearly confluent plaques was harvested by scraping into a 50-ml conical tube, subjected to a vortex procedure, and centrifuged at  $5,000 \times g$  for 10 min. Supernatants were treated with 25  $\mu\text{g/ml}$  DNase I before being passed through a 0.45- $\mu\text{m}$ -pore-size syringe filter and stored at 4°C.

Recipient cells were grown to stationary phase in 2 ml TY broth at 37°C. Cells (0.9 ml) were mixed with 5  $\mu\text{l}$  SPP1 donor phage stock. TY broth (9 ml) was added to the mixture and allowed to stand at 37°C for 30 min. Transduction mixtures were centrifuged at  $5,000 \times g$  for 10 min, supernatants were discarded, and pellets were resuspended in the remaining volume. Cell suspensions (100  $\mu\text{l}$ ) were plated on TY fortified with 1.5% agar, the appropriate antibiotic, and 10 mM sodium citrate.

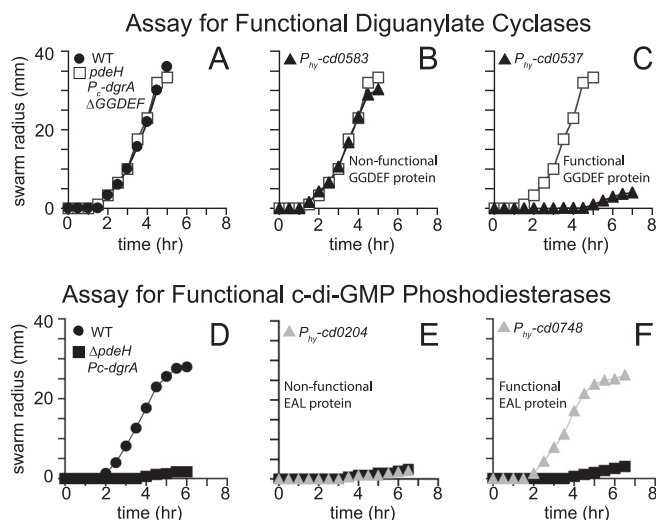
**Swarm expansion assay (57).** *Bacillus subtilis* strains were streaked on LB plates with the proper antibiotics (see the supplemental material) and allowed to grow overnight at 37°C. A single colony was used to inoculate a 2-ml LB culture, which was grown overnight at room temperature. The following morning, 150  $\mu\text{l}$  stationary culture was used as an inoculum for 3 ml LB broth cultures containing 1 mM IPTG (isopropyl- $\beta$ -D-thiogalactopyranoside) and antibiotics. Cells were grown to mid-log phase (optical density at 600 nm [ $\text{OD}_{600}$ ] of 0.4 to 0.8) at 37°C and resuspended to an  $\text{OD}_{600}$  of 10 in phosphate-buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 10 mM  $\text{Na}_2\text{HPO}_4$ , and 2 mM  $\text{KH}_2\text{PO}_4$ , pH 8.0) containing 0.5% India ink (Higgins). Freshly prepared LB containing 0.7% Bacto agar (25 ml/plate) was dried for 10 min in a laminar flow hood, centrally inoculated with 10  $\mu\text{l}$  of the cell suspension, dried for another 10 min, and incubated at 37°C. The India ink demarked the origin of the colony, and the swarm radius was measured relative to the origin. For consistency, an axis was drawn on the back of the plate and measurements of swarm radii were taken along this transect (57).

**Fluorescence-activated cell sorter (FACS) analysis.** *B. subtilis* strains were streaked out on LB plates containing 100  $\mu\text{g/ml}$  spectinomycin and allowed to grow overnight at 37°C. A single colony was then picked for inoculation of 2 ml LB containing 1 mM IPTG. After 3 h at 37°C, 20  $\mu\text{l}$  culture was diluted into 1 ml PBS, and samples were analyzed using a BD LSR II flow cytometer (BD Biosciences) with excitation at 488 nm. Results were analyzed using FloJo software (TreeStar Inc.).

## RESULTS

***Bacillus subtilis* swarming motility as a platform to identify active c-di-GMP metabolic enzymes.** To examine c-di-GMP signaling in *B. subtilis*, we previously engineered a *dgc* and *pde* null mutant with an additional constitutively expressed copy of the c-di-GMP receptor *dgrA* ( $\Delta ydaK \Delta dgcK \Delta dgcW dgcP::tet pdeH::kan amyE::Pc-dgrA spec$  [NPS236]) (43, 50). The resulting strain is devoid of c-di-GMP metabolic enzymes and shows swarming motility indistinguishable from the wild-type motility (Fig. 2A). Further, overproduction of at least two heterologous proteins that produce c-di-GMP in this background robustly inhibited swarming in a manner dependent on the presence of the DgrA c-di-GMP receptor (43). In this c-di-GMP null background, we examined the activity of 18 full-length, nondegenerate GGDEF proteins and the single putative bifunctional, nondegenerate GGDEF and EAL protein from *C. difficile* 630 (Fig. 1A and C). All coding sequences were constructed as a translational fusion to the *B. subtilis* *dgcP* leader to ensure proper transcription and translation initiation in the heterologous host and inserted into the *B. subtilis* *thrC* locus. Twelve of 19 putative DGCs tested were capable of inhibiting swarming motility, indicative of active DGCs possessing the ability to produce c-di-GMP (Fig. 2A to C; see also Fig. S2 in the supplemental material).

Given the clear, robust motility phenotype in the engineered strain used to test for DGC activity, we proposed that a

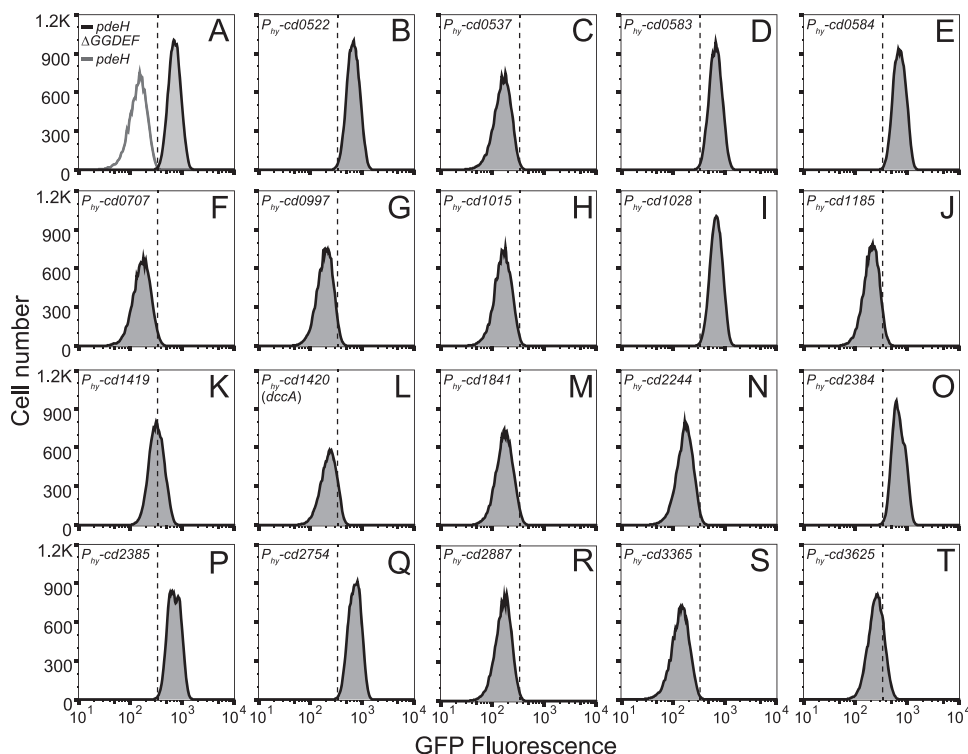


**FIG 2** Swarm expansion assays for engineered *B. subtilis* strains expressing GGDEF or EAL genes from *C. difficile* 630. Each point represents an average of three replicates. (A to C) Open squares indicate swarming motility of parent strain NPS236 (A), whereas filled triangles depict swarming motility for an inactive (B) or active (C) diguanylate cyclase. Assays to examine GGDEF protein activity were conducted in a background with constitutive expression of *dgrA* (*Pc-dgrA*) and mutated for *pdeH* and endogenous GGDEF-encoding genes. (D to F) Filled squares indicate swarming motility of parent strain NPS235 (D), whereas gray triangles depict swarming motility for an inactive (E) or active (F) c-di-GMP phosphodiesterase(s). Assays to examine EAL protein activity were conducted in a background with constitutive expression of *dgrA* (*Pc-dgrA*) and mutated for *pdeH*. A comprehensive data set assessing swarming motility of the 37 putative c-di-GMP metabolic enzymes from *C. difficile* is shown in Fig. S2 and S3 in the supplemental material.

complementary strain could be constructed to test for c-di-GMP phosphodiesterase activity. With this goal in mind, we generated a strain mutated for the primary c-di-GMP phosphodiesterase *pdeH* while carrying an additional constitutively expressed copy of c-di-GMP receptor *dgrA* (*pdeH::kan amyE::Pc-dgrA spec* [NPS235]) (43). Abrogation of PdeH activity results in elevated levels of c-di-GMP, and thus this strain shows inhibited motility (Fig. 2D). Thus, introduction of a sufficiently active c-di-GMP phosphodiesterase into this background should deplete c-di-GMP and coordinately restore motility. To test this hypothesis, the 19 EAL domain proteins from *C. difficile* 630 were expressed under the control of an IPTG-inducible *P<sub>hyspank</sub>* promoter as a translational fusion to the *B. subtilis* *dgcP* leader and inserted into the *B. subtilis* *thrC* locus (Fig. 1B and C). Twelve of the 19 putative PDEs tested restored motility, indicative of active PDEs with the ability to degrade c-di-GMP (Fig. 2D to F; see also Fig. S3 in the supplemental material).

**Engineering a riboswitch-based fluorescence reporter to identify active c-di-GMP metabolic enzymes.** As motility gave an all-or-none phenotype, we developed a complementary method to measure variations in c-di-GMP levels by adapting a natural c-di-GMP riboswitch. Riboswitches are *cis*-acting RNA elements generally located at the 5' untranslated region (5'-UTR) of mRNAs that can alter gene expression by sensing metals, metabolites, or secondary messenger molecules (58–63). In response to ligand binding to a riboswitch aptamer, changes occur in the expression platform that result in regulation of downstream open reading frames. A c-di-GMP-responsive “off switch” from *B. cereus*





**FIG 3** Riboswitch-based assessment of *in vivo* activity for *C. difficile* GGDEF protein-encoding genes expressed in engineered *B. subtilis* strains. Panels show representative histograms with cell count versus GFP fluorescence of strains expressing the indicated gene. (A) Fluorescence of cells containing a constitutively expressed c-di-GMP-responsive riboswitch-GFP reporter in strains mutated for *pdeH* alone (NPS400) or in conjunction with all DGCs ( $\Delta$ GGDEF; NPS401) serves as a control for reporter response levels in the presence or absence of c-di-GMP, respectively. A vertical line representing the histogram boundaries between control strains is shown in all panels as a reference. (B to T) Cell-based fluorescence histograms of derivative strains that express the indicated gene from an IPTG-inducible *P<sub>hypank</sub>* promoter (Phy) in the presence of 1 mM IPTG (NPS402 to NPS420) are shown. Decreases in GFP fluorescence relative to that of the parent strain (shaded in panel A) are indicative of an active diguanylate cyclase. All experiments were done in triplicate; statistical analysis of mean fluorescence is included in Fig. 5.

termed GEMM1 has been characterized previously, and we cloned the GEMM1 aptamer, flanked by the *B. cereus* M-box riboswitch expression platform, including its intrinsic transcriptional terminator, upstream of the coding sequence for GFP (39, 55).

As designed, this chimeric M-box/GEMM riboswitch reporter responds to elevation of c-di-GMP levels by increasing the frequency of transcriptional termination upstream of the GFP coding sequence, thereby decreasing the steady-state levels of GFP. To test the functionality of this reporter, we introduced the construct into the *B. subtilis amyE* gene in either a c-di-GMP null ( $\Delta$ *ydaK*  $\Delta$ *dgcK*  $\Delta$ *dgcW* *dgcP::tet pdeH::kan amyE::Pmbox-bcl<sub>1</sub>GEMM-GFP spec* [NPS401]) or an elevated c-di-GMP (*pdeH::kan amyE::Pmbox-bcl<sub>1</sub>GEMM-GFP spec* [NPS400]) background. Cells were grown at in LB for 3 h and subjected to flow cytometry analysis to assess GFP fluorescence. As predicted for a c-di-GMP-responsive reporter, the average cell GFP fluorescence was highest in the c-di-GMP null strain and decreased in the presence of c-di-GMP (Fig. 3A).

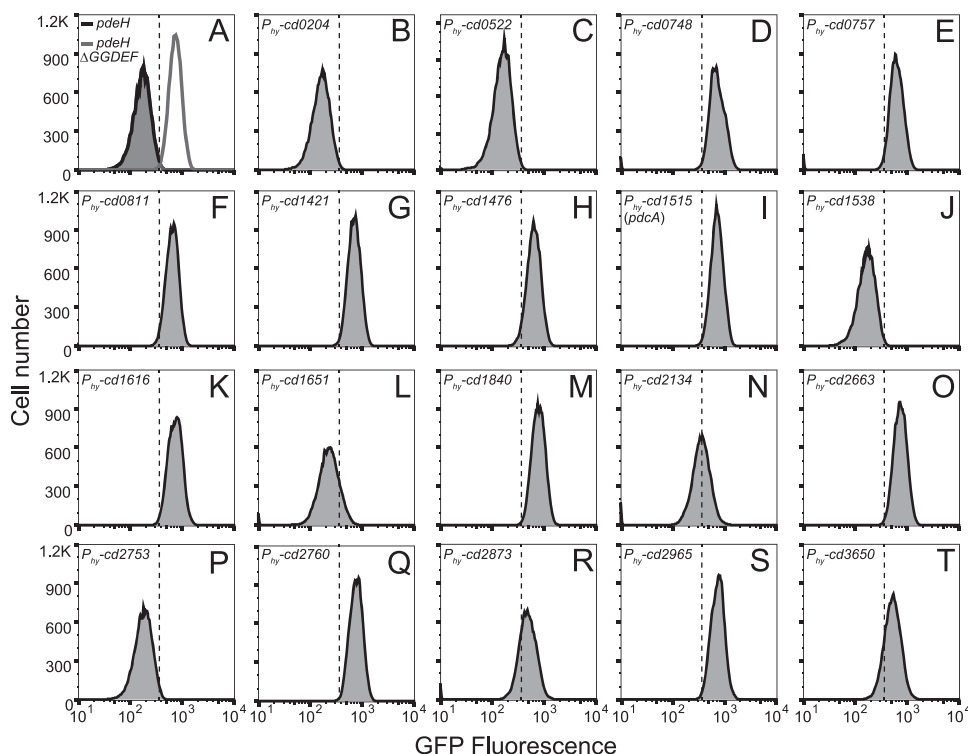
Having constructed a c-di-GMP-responsive fluorescence reporter, we next introduced the 37 *C. difficile* genes tested in swarming motility assays (Fig. 1) into the appropriate background containing the riboswitch-based reporter expression cassette. All putative DGCs (Fig. 1A) were introduced into the NPS401 c-di-GMP null reporter strain, whereas putative PDEs (Fig. 1B) were introduced into c-di-GMP elevated strain NPS400. The single

gene product harboring putative DGC and PDE domains (Fig. 1C) was introduced into both NPS401 and NPS400. Each single gene expression strain was analyzed for GFP fluorescence by flow cytometry and compared to its parent strain to define active DGCs and PDEs. From these data, 12 of 19 putative DGCs were shown to be active as indicated by a decrease in reporter fluorescence relative to that seen with the parent strain (Fig. 3). Conversely, 15 of 19 putative PDEs were shown to be active on the basis of an increase in reporter fluorescence relative to that seen with the parent strain, in excellent agreement with our swarming motility data (Fig. 4).

## DISCUSSION

In this work, we demonstrated the robust ability of engineered *B. subtilis* strains to serve as heterologous hosts to screen for active diguanylate cyclases and c-di-GMP phosphodiesterases on the basis of distinct systems that respond to changes in c-di-GMP levels via alterations in swarming motility or fluorescence of a riboswitch reporter. Our swarming motility assays rely on binding of c-di-GMP to the DgrA receptor to inhibit motility, whereas the riboswitch reporter assays depend upon direct sensing of c-di-GMP to effect change in the total GFP fluorescence.

Through comparison of swarming motility and riboswitch fluorescence data, we noted that active DGCs are reliably detected with either system (Fig. 5A; see also Fig. S2 and S3 in the supplemental material). Even modest levels of c-di-GMP production, as



**FIG 4** Riboswitch-based assessment of *in vivo* activity for *C. difficile* EAL protein-encoding genes expressed in engineered *B. subtilis* strains. Panels show histograms with cell count versus GFP fluorescence of strains expressing the indicated gene. (A) Fluorescence of cells containing a constitutively expressed c-di-GMP-responsive riboswitch-GFP reporter in strains mutated for *pdeH* alone (NPS400) or in conjunction with all DGCs ( $\Delta$ GGDEF; NPS401) serves as a control for reporter response levels in the presence or absence of c-di-GMP, respectively. A vertical line representing the histogram boundaries between control strains is shown in all panels as a reference. (B to T) Cell-based fluorescence histograms of derivative strains that express the indicated gene from an IPTG-inducible *P<sub>hyspank</sub>* promoter (Phy) in the presence of 1 mM IPTG (NPS421 to NPS439) are shown. Increases in GFP fluorescence relative to that of the parent strain (shaded in panel A) are indicative of an active c-di-GMP phosphodiesterase. All experiments were done in triplicate; statistical analysis of mean fluorescence is included in Fig. 5.

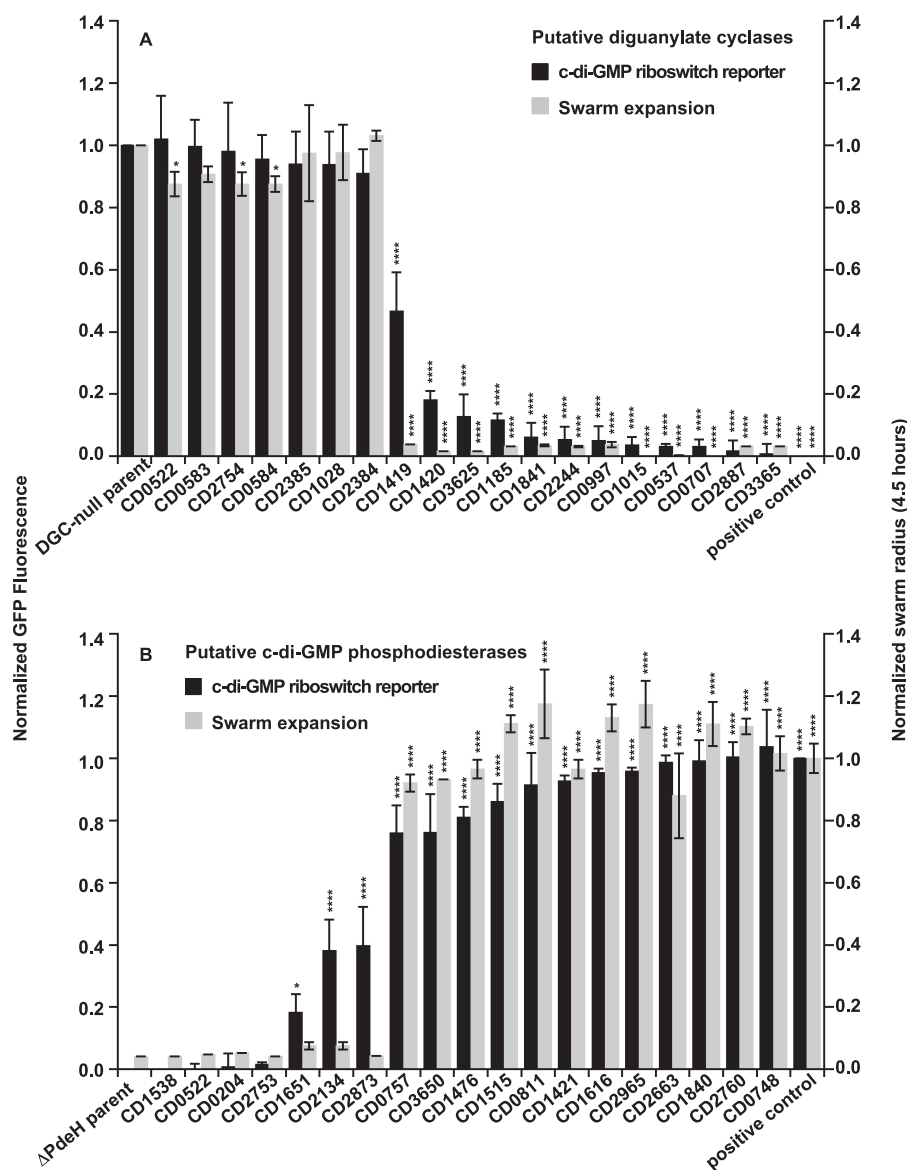
seen via an intermediate level of GFP fluorescence for the riboswitch reporter (e.g., CD1419) (Fig. 5A), result in strong inhibition of swarming motility. In our study of putative PDEs, both assays were again sufficient to identify the most active enzymes. However, given that low levels of c-di-GMP are sufficient to inhibit swarming motility, a partial depletion of c-di-GMP pools by an active PDE(s) may not restore swarming motility (see CD1651, CD2134, and CD2873 data in Fig. 5B; see also Fig. S3 in the supplemental material). Conversely, moderately active PDEs, those capable of converting only a fraction of the active PDE pool to pGpG, presented as active enzymes in the riboswitch reporter measurements (Fig. 4 and 5B). Taking the results together, utilization of biological outputs such as biofilm formation and motility may be best suited to identifying active DGCs whereas screens for active PDEs using a biological phenotype may result in a subset of false negatives owing to the inability of all active PDEs to sufficiently deplete c-di-GMP.

Our data can be compared to data from previous reports in which putative *C. difficile* c-di-GMP metabolic enzymes have been examined (45, 46, 64). In particular, a comprehensive study by Bordeleau et al. (45) studied the effects of heterologous expression of *C. difficile* genes on motility in Gram-negative *V. cholerae*. Our data largely correlate with that of the prior report, but our study appeared to be more sensitive and identified three additional active DGCs (CD0537, CD2887, and CD3365) and four additional active PDEs (CD0748, CD0811, CD1421, and CD2134). While the

ability to identify additional active c-di-GMP metabolic enzymes using the Gram-positive *B. subtilis* is significant, the comparison of our *B. subtilis* systems to the *V. cholerae* study is best used to highlight an important challenge of studying c-di-GMP metabolic enzymes: the environmental context is paramount. Care must be taken to choose a suitable host, with the understanding that variables, including environmental stimuli, host protein factors, and protein folding—to name but a few—may impact the ability to identify active enzymes.

The *B. subtilis* strains employed in this work have many benefits for use as heterologous hosts that could mitigate many of the aforementioned concerns while providing an opportunity for further understanding of c-di-GMP metabolic enzymes. Specifically, the *B. subtilis* hosts were engineered to contain a minimal set of c-di-GMP signaling components, reducing or eliminating the possibility of indirect changes in c-di-GMP resulting from endogenous signaling. Furthermore, *B. subtilis* is a safe, easy-to-culture, nonpathogenic host with a wide array of genetic techniques available to adapt for subsequent studies. As an example, additional genes could be introduced to screen for modulators of either active or inactive DGCs or PDEs on the basis of motility or fluorescence.

Both motility assays and riboswitch reporter measurements rely on routine techniques with high reproducibility while exhibiting a clear distinction between active and inactive enzymes. In comparisons of our two systems, the riboswitch reporter may



**FIG 5** Comparative analyses of riboswitch-based c-di-GMP GFP reporter and swarm radii for putative *C. difficile* 630 diguanylate cyclases (A) and c-di-GMP phosphodiesterases (B). Black bars show the normalized mean values of GFP fluorescence using a riboswitch-based c-di-GMP reporter, while gray bars show normalized swarm radii at 4.5 h, with standard deviations indicated for all data. Data were subjected to one-way analysis of variance (ANOVA) using Dunnett's multiple-comparison test with at least three measurements for each data point using Prism 6 software. Fluorescence data are normalized to same-day control strains in each experimental set. Strains are sorted on the basis of the increasing difference in the mean GFP fluorescence level relative to that of the parent strain. As such, proteins that alter c-di-GMP levels, i.e., active enzymes, are on the right in each panel. \*,  $P$  value < 0.05; \*\*\*\*,  $P$  value < 0.0001.

have an advantage in that it identifies moderately active c-di-GMP phosphodiesterases whereas the two systems similarly identify active diguanylate cyclases. In practice, the swarming motility assays are perhaps more accessible, having no requirements for either flow cytometry or, alternatively, a suitable fluorescence microscope. In conclusion, the systems developed in this study to survey the activity of putative c-di-GMP metabolic enzymes should significantly impact our understanding of the switch between bacterial lifestyles and guide the subsequent development of small-molecule modulators of bacterial motility, biofilm formation, and virulence by providing a rapid assessment of predicted c-di-GMP signaling components from any exogenous organism.

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