



Single-Cell Microscopy Reveals That Levels of Cyclic di-GMP Vary among *Bacillus subtilis* Subpopulations

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ABSTRACT The synthesis of signaling molecules is one strategy bacteria employ to sense alterations in their environment and rapidly adjust to those changes. In Gram-negative bacteria, bis-(3'-5')-cyclic dimeric GMP (c-di-GMP) regulates the transition from a unicellular motile state to a multicellular sessile state. However, c-di-GMP signaling has been less intensively studied in Gram-positive organisms. To that end, we constructed a fluorescent *yfp* reporter based on a c-di-GMP-responsive riboswitch to visualize the relative abundance of c-di-GMP for single cells of the Gram-positive model organism *Bacillus subtilis*. Coupled with cell-type-specific fluorescent reporters, this riboswitch reporter revealed that c-di-GMP levels are markedly different among *B. subtilis* cellular subpopulations. For example, cells that have made the decision to become matrix producers maintain higher intracellular c-di-GMP concentrations than motile cells. Similarly, we find that c-di-GMP levels differ between sporulating and competent cell types. These results suggest that biochemical measurements of c-di-GMP abundance are likely to be inaccurate for a bulk ensemble of *B. subtilis* cells, as such measurements will average c-di-GMP levels across the population. Moreover, the significant variation in c-di-GMP levels between cell types hints that c-di-GMP might play an important role during *B. subtilis* biofilm formation. This study therefore emphasizes the importance of using single-cell approaches for analyzing metabolic trends within ensemble bacterial populations.

IMPORTANCE Many bacteria have been shown to differentiate into genetically identical yet morphologically distinct cell types. Such population heterogeneity is especially prevalent among biofilms, where multicellular communities are primed for unexpected environmental conditions and can efficiently distribute metabolic responsibilities. *Bacillus subtilis* is a model system for studying population heterogeneity; however, a role for c-di-GMP in these processes has not been thoroughly investigated. Herein, we introduce a fluorescent reporter, based on a c-di-GMP-responsive riboswitch, to visualize the relative abundance of c-di-GMP for single *B. subtilis* cells. Our analysis shows that c-di-GMP levels are conspicuously different among *B. subtilis* cellular subtypes, suggesting a role for c-di-GMP during biofilm formation. These data highlight the utility of riboswitches as tools for imaging metabolic changes within individual bacterial cells. Analyses such as these offer new insight into c-di-GMP-regulated phenotypes, especially given that other biofilms also consist of multicellular communities.

KEYWORDS *Bacillus subtilis*, riboswitch, biofilm, cyclic di-GMP, microscopy

Bacteria use diverse strategies to sense alterations in their environment and rapidly adjust to those changes. Adaptability to cell stresses can sometimes be manifested as phenotypic variation, where individual cells differentially express subsets of their genes, sometimes occurring within an isogenic population (1–3). For example, in the Gram-positive model microorganism *Bacillus subtilis*, bistability in gene expression can

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help guide differentiation of distinct cell types (4, 5). Some cells within a bulk *B. subtilis* population express motility genes, as induced by the sigma factor SigD (6). Simultaneously, a smaller subset are activated by the ComK transcription factor to differentiate into competent cells, proficient in DNA uptake and primed for homologous recombination (7). Other cells within the population are triggered by regulatory proteins (e.g., SinR/SinI, SlrR, and DegU) to differentiate into sessile chains that overproduce biofilm matrix components or extracellular proteases (8–11). Yet another subpopulation is irreversibly directed into the developmental program of endospore formation, which culminates in the formation of a dormant cell type resistant to many stresses (12, 13). For cells that will be encouraged into endospore formation, nutrient limitation stimulates a phosphorelay that results in the phosphorylation of the master regulator Spo0A, which triggers the onset of sporulation. Furthermore, cells that are further into the sporulation program than others will secrete extracellular killing factors to cannibalize their siblings for nutrients (14). In sum, *B. subtilis* is capable of switching between cell fates through highly coordinated processes that are governed by master regulators, two-component systems, and phosphorylation cascades.

Signaling molecules also influence *B. subtilis* differentiation pathways. Several types of signaling molecules have been discovered that coordinate gene expression and regulate bacterial behavior. One class of extracellular signaling molecules, called autoinducers, regulate quorum sensing and allow bacterial populations to behave collectively (15). In addition to autoinducers, a variety of nucleotide-based second messengers are specifically produced for intracellular signaling in bacteria (16–18). One particular ribonucleotide signaling molecule, bis-(3'-5')-cyclic dimeric GMP (c-di-GMP), has been shown in many bacteria to regulate the transition from a unicellular motile state to a multicellular sessile community (19, 20). c-di-GMP signaling networks are harbored by almost every phylum in the bacterial domain, making this molecule a near-universal second messenger, although it has still been incompletely examined in several model microbes (21).

In response to external stimuli, c-di-GMP is synthesized from 2 GTP molecules by GGDEF domain-containing diguanylate cyclases (DGCs) (22–28). The second messenger can then bind intracellular effectors to direct physiological changes. EAL or HD-GYP domain-containing phosphodiesterases (PDEs) hydrolyze cyclic di-GMP into the linear dinucleotide 5'-phosphoguanlyl-(3'-5')-guanosine (pGpG) (29–38), which is then recycled into nucleoside monophosphate pools through the action of oligoribonucleases (39–41)). Through bioinformatic analyses, GGDEF, EAL, and HD-GYP domain-containing enzymes have readily been identified in almost all bacterial phyla (35, 42). Conversely, there is greater diversity in the classes of receptors that associate with c-di-GMP. These receptors often have no sequence or structural similarity to one another, making the prediction of c-di-GMP receptors sometimes difficult (43). In addition to the large number of protein receptors, riboswitches also have been shown to bind c-di-GMP to regulate gene expression (44, 45). Riboswitches are located in the untranslated regions of mRNA transcripts and coordinate downstream gene expression in response to highly specific interactions with their cognate ligand (46). The presence of c-di-GMP-responsive riboswitches upstream of a diverse array of genes, such as those encoding GGDEF/EAL/HD-GYP proteins, flagella, pili, other motility factors, transcription factors, and membrane transporters, suggests many different targets of c-di-GMP regulation in bacteria (47, 48).

B. subtilis harbors three DGCs (DgcK, DgcP, and DgcW), one PDE (PdeH), and three putative c-di-GMP receptors (MotI, YdaK, and YkuI) (see Fig. S1 in the supplemental material) (49, 50). MotI is a PilZ domain-containing protein that is thought to inhibit flagellar motility by acting as a molecular clutch on the flagellar stator element MotA, to disengage and sequester it from the flagellar rotor FliG (51). Genetic evidence also supports a direct relationship between c-di-GMP and the regulation of motility through MotI and PdeH (49, 50). While deletion of *pdeH* results in a swarming motility defect, it does not result in any obvious changes to biofilm colonies or pellicles. Overexpression of the DGCs also did not appear to affect biofilms, initially suggesting that c-di-GMP is

not important for the control of biofilm formation in this organism. Recently, however, data suggest that the degenerate DGC YdaK is a c-di-GMP receptor that is likely to participate in biofilms. YdaK is encoded within the *ydaJKLMN* operon. While the exact role of the *yda* operon has yet to be determined, the operon may be involved in the synthesis of an unknown exopolysaccharide, which may bolster the stress resistance of the biofilm under specific physiological conditions (52, 53). Therefore, more remains to be understood regarding the role of c-di-GMP in regulating *B. subtilis* biofilm formation. The third putative receptor, Ykul, is a catalytically inactive PDE that has retained the ability to bind c-di-GMP but not hydrolyze it (54). Interestingly, its deletion was shown to confer resistance to inhibitory concentrations of zinc (55). It is possible that deletion of *ykul* somehow restricts access to zinc, although a mechanistic model for this phenotype has yet to be identified. Furthermore, its involvement in the regulation of lifestyle switching in *B. subtilis* has not yet been ascertained.

The genetic malleability and wealth of knowledge regarding *B. subtilis* development make it an excellent experimental model in which to interrogate the contribution of c-di-GMP regulation to cell differentiation in *Firmicutes*. Elevated c-di-GMP levels have been shown to inhibit motility in this organism (49, 50); however, it is currently unknown if sporulation and competence are also regulated by this signaling molecule. Developing evidence suggests that c-di-GMP may also affect the composition of extracellular polysaccharides during biofilm formation in this organism (52, 53). Yet, the methods used to examine whether c-di-GMP affects biofilm formation have thus far incompletely considered that *B. subtilis* biofilms are heterogeneously composed of several subpopulations (4, 5, 56). In this study, we made use of a fluorescent c-di-GMP-responsive riboswitch reporter and quantified its expression *in vivo* for single cells of *B. subtilis*. When combined with fluorescent transcriptional reporters that demarcate the primary classes of cell types, the c-di-GMP riboswitch reporter displayed markedly different levels of fluorescence within the multicellular community. The combination of these data revealed that within a single population, some cell fates correlate with high levels of c-di-GMP while other fates exhibit low levels of c-di-GMP. Therefore, these data demonstrate that for some bacteria, c-di-GMP levels are adjusted heterogeneously across bulk populations. Finally, these data highlight the utility of riboswitches as tools for imaging metabolic changes within individual bacterial cells.

RESULTS

Analysis of a c-di-GMP-responsive riboswitch upstream of *Bacillus licheniformis* lichenysin synthesis genes. In this study, we sought to identify a riboswitch that could be used to construct a genetic reporter of c-di-GMP. A bioinformatics-based search for c-di-GMP-responsive riboswitches in *Bacillales* revealed a particularly interesting candidate in the untranslated leader region of the *Bacillus licheniformis* *lch* gene cluster. This nearly 27-kb operon (*lchA*) encodes the subunits of the nonribosomal peptide synthetase that makes lichenysin. Lichenysin is an antimicrobial cyclic lipopeptide that is virtually identical to surfactin, an important specialized metabolite produced by *B. subtilis* (57–59). The location of this riboswitch is particularly interesting given that riboswitches have not been previously analyzed as being important for the genetic regulation of secondary metabolites. We chose to further examine this class I riboswitch to determine whether it is a good candidate for the development of a genetic reporter of c-di-GMP abundance.

The interaction between a riboswitch and its cognate ligand induces a conformational change in the transcript that modulates downstream gene expression (60). Many riboswitches couple the detection of their target signal to transcription attenuation. In the unbound state, these riboswitches will oftentimes adopt a conformation in which an “antiterminator” helix is created from the left half of the terminator and an upstream sequence. Then, when bound to its cognate ligand, the riboswitch will adopt an alternate conformation that allows for the formation of an intrinsic terminator (61), causing disassociation of the transcription elongation complex (Fig. 1A). Manual inspection of the *lchAA* putative riboswitch revealed that its sequence included a

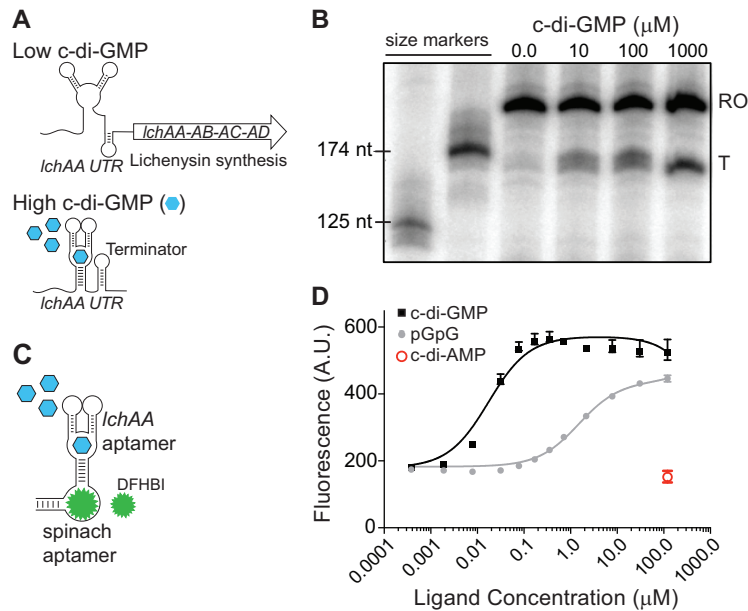


FIG 1 The *LchAA* riboswitch promotes transcription termination in response to c-di-GMP. (A) Schematic of the mechanism of regulation by the *B. licheniformis* *LchAA* riboswitch in response to high and low levels of c-di-GMP. (B) A transcription termination assay of the *LchAA* untranslated region (UTR) RNA with increasing concentrations of c-di-GMP (0 to 1,000 μM) shows premature termination of runoff (RO) within the leader sequence compared to that of a terminated (T) transcript. (C) Schematic of the Spinach2 RNA allosterically regulated by the *LchAA* riboswitch aptamer in response to c-di-GMP. (D) *In vitro* fluorescence assay of the riboswitch-Spinach2 RNA (100 nM) in the presence of saturating DFHBI (10 μM) and increasing concentrations of c-di-GMP or pGpG. Binding affinity measurements are representative of two independent replicates.

candidate terminator site. Therefore, to determine if c-di-GMP modulates downstream gene expression of the putative *B. licheniformis* riboswitch, we performed a transcription termination assay *in vitro* (Fig. 1B). A DNA template of the *LchAA* untranslated element that included 50 nucleotides beyond the putative terminator ("runoff") was generated by PCR and mixed with RNA polymerase holoenzyme in the presence of various amounts of c-di-GMP. Under the conditions that we used, a concentration of 10 μM c-di-GMP began to promote transcription termination at the terminus of the riboswitch, and ligand-responsive termination continued to increase up to the maximum concentration tested, 1 mM c-di-GMP.

To directly measure binding of c-di-GMP to the *LchAA* riboswitch, we identified the *LchAA* aptamer domain via manual inspection and fused it to the Spinach2 aptamer. Spinach and Spinach2 RNAs fluoresce upon binding 3,5-difluoro-4-hydroxybenzylidene imidazolinone (DFHBI) (62, 63). Spinach variants can be carefully fused to riboswitch aptamer domains such that the Spinach domain fluoresces only in response to binding of the riboswitch ligand (64–66). Therefore, we isolated the *LchAA* aptamer and fused it to the P2 stem-loop of Spinach2 (see Fig. S2 in the supplemental material). We hypothesized this would create an allosteric version of Spinach2, thereby creating an RNA with two distinct binding sites, for which binding of the *LchAA* aptamer to its cognate ligand will then trigger binding of DFHBI to Spinach2 (Fig. 1C). We then measured Spinach2 fluorescence in the presence of saturating concentrations of DFHBI and with increasing concentrations of c-di-GMP. This revealed that fluorescence of the *LchAA* aptamer-Spinach2 RNA was dependent on an appropriate amount of c-di-GMP, confirming that the *LchAA* aptamer acts as a sensor of c-di-GMP. In contrast, we did not detect Spinach2 fluorescence in the presence of 100 μM c-di-AMP. The *LchAA* aptamer, as part of a Spinach2 biosensor construct, bound c-di-GMP with an apparent dissociation constant (K_d) of 17 nM. While this apparent K_d is lower than the concentration of c-di-GMP required to promote transcription termination *in vitro* on purified DNA

templates, it has been observed that many riboswitches are not driven by equilibrium ligand interactions. Instead, many riboswitches are driven by coordination of the kinetics of ligand association and RNA polymerization speed (67). In these instances, a higher concentration of c-di-GMP is required to promote a conformational change of the expression platform *in vitro*. Fluorescence of the *lchAA*-Spinach2 aptamer was also observed in the presence of pGpG, the linear dinucleotide and c-di-GMP degradation product; however, the apparent K_d for this molecule was 2 orders of magnitude higher than that for c-di-GMP (Fig. 1D). This roughly agrees with previous results in which pGpG binds a c-di-GMP riboswitch with much poorer affinity (68). We conclude from these aggregate data that c-di-GMP specifically interacts with the *lchAA* riboswitch to promote transcription attenuation of the *lchAA* operon.

A c-di-GMP riboswitch-*yfp* reporter is heterogeneously expressed in *B. subtilis*.

We next wanted to assess c-di-GMP levels in single cells of *B. subtilis* *in vivo*. Since intracellular expression of Spinach has not been examined for this organism, we first ectopically integrated a constitutively expressed Spinach2 sequence into the *B. subtilis* PY79 genome at a nonessential locus. When cells reached late log phase (optical density at 600 nm [OD₆₀₀] of 1.0), they were incubated with saturating DFHBI for 1 h and examined by fluorescence microscopy. These cells exhibited a roughly 2-fold increase in fluorescence compared to that of untreated cells. However, many cells remained fully inactivated for fluorescence in the presence of DFHBI, indicating broad heterogeneity in fluorescence by a constitutive Spinach2 reporter (see Fig. S3). Incubation with DFHBI for 1 h had no apparent effect on bacterial growth, either for cells in culture or upon examination by microscopy.

As the Spinach2-based reporter was unacceptably nonuniform in *B. subtilis*, we constructed a reporter based on expression of yellow fluorescent protein (YFP). We ectopically integrated a constitutively expressed *yfp* sequence into the *B. subtilis* genome and analyzed cells at late log phase (OD₆₀₀ of 1.0) by fluorescence microscopy. Unlike the strain that constitutively expressed Spinach2, the constitutive *yfp* strain exhibited more-uniform fluorescence. This population exhibited a unimodal distribution of fluorescence per cell (Fig. 2A and B). We then constructed a corresponding reporter based on the c-di-GMP riboswitch. The entire leader sequence of *B. licheniformis lchAA* was inserted between the constitutively active promoter sequence and *yfp*. Unlike the constitutive *yfp* reporter, a strain containing the riboswitch-*yfp* reporter exhibited two prominently distinct levels of fluorescence within the population. Nearly 70% of the cells were moderately fluorescent compared to the constitutive *yfp* strain, while the remaining cells exhibited significantly diminished fluorescence (Fig. 2A and B). Therefore, unlike the unimodal distribution of fluorescence exhibited by the constitutive *yfp* reporter, the riboswitch-*yfp* reporter strain appeared to show a bimodal distribution of fluorescence. To determine whether this distribution was due to riboswitch regulation of YFP, we mutated the riboswitch element. Specifically, we deleted 65 nucleotides from the 5' end of the *lchAA* leader, which includes part of the c-di-GMP-binding aptamer, and reexamined cellular fluorescence. This population of cells uniformly expressed YFP at levels similar to that of the constitutive *yfp* reporter construct (Fig. 2A and C). Long considered a *B. subtilis* "legacy" laboratory strain, PY79 has been widely used in a plethora of genetic analyses that have provided profound insight into the molecular pathways that govern cellular differentiation. Indeed, the regulation of cell-specific gene expression in *B. subtilis* was determined in this genetic background (69–74). Historically, however, undomesticated strains such as 3610 have often been used to study biofilm formation (10, 75–77). We therefore also assessed our riboswitch-*yfp* reporter in the undomesticated 3610 background. We observed an identical distribution of fluorescence to the PY79 background, suggesting that c-di-GMP dynamics behave similarly in both strains (see Fig. S4). Therefore, we chose to continue our studies with PY79 for interrogating gene expression differences between different cellular subtypes. Taken together, these findings suggest that the c-di-GMP riboswitch-*yfp* reporter is bimodally expressed in a population of *B. subtilis*.

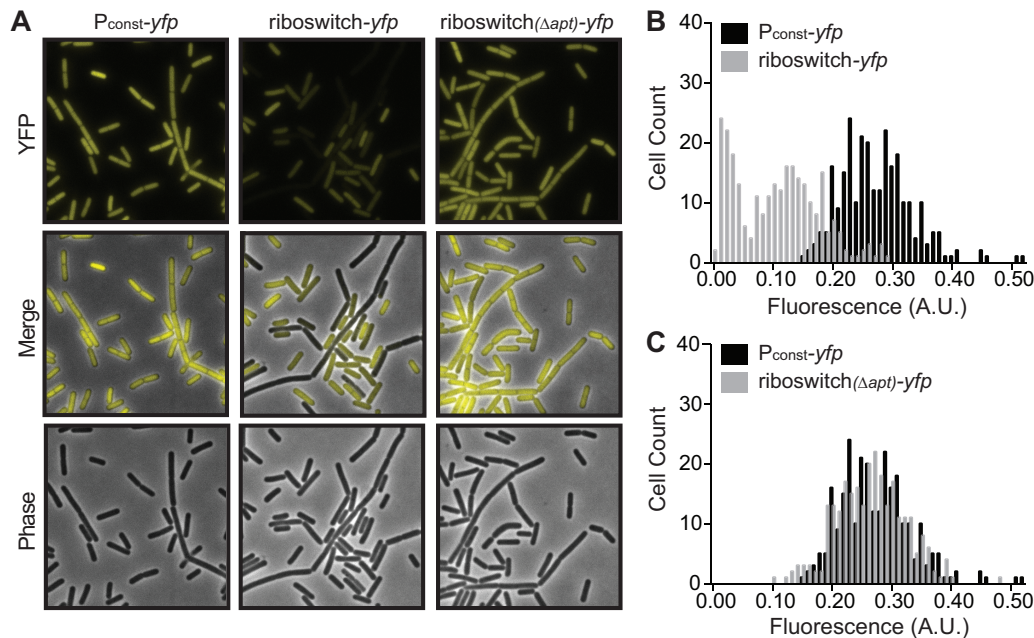


FIG 2 Expression of a *lchAA* riboswitch-*yfp* reporter *in vivo* results in bimodal distribution of fluorescence. (A) Representative microscopy images of wild-type *B. subtilis* PY79 expressing a constitutive $P_{const}\text{-yfp}$ reporter, a riboswitch reporter construct $P_{const}\text{-riboswitch}\text{-yfp}$, or a riboswitch reporter construct comprising a deletion corresponding to the riboswitch aptamer ($P_{const}\text{-riboswitch}^{\Delta\text{apt}}\text{-yfp}$). (B and C) Histograms of the quantification of fluorescence intensity per cell of wild-type *B. subtilis* PY79 expressing $P_{const}\text{-yfp}$ compared to $P_{const}\text{-riboswitch}\text{-yfp}$ (B) or $P_{const}\text{-yfp}$ compared to $P_{const}\text{-riboswitch}^{\Delta\text{apt}}\text{-yfp}$ (C) ($n \approx 300$).

The riboswitch reduces downstream gene expression in response to elevated c-di-GMP. Of the two phosphodiesterases encoded in the *B. subtilis* genome, previous work has shown only that encoding PdeH to be active (49, 50). With this in mind, we wanted to confirm that the bimodal distribution of fluorescence observed in single cells of *B. subtilis* was solely due to differences in intracellular c-di-GMP levels. To do this, we integrated the constitutive *yfp* and *riboswitch-yfp* reporter fusions into a ΔpdeH knock-out strain. We predicted that loss of PdeH should inhibit hydrolysis of c-di-GMP and consequently elevate intracellular levels, which should promote premature transcription termination in the *riboswitch-yfp* reporter fusion and thereby reduce the YFP signal. Indeed, the *riboswitch-yfp* reporter resulted in significantly decreased *yfp* expression in the ΔpdeH background, suggesting intracellular cyclic di-GMP was elevated in all cells at late log phase (OD_{600} of 1.0) (Fig. 3B and D). Conversely, constitutive *yfp* expression was unaffected by the ΔpdeH mutation, confirming the specificity of the *riboswitch-yfp* reporter for c-di-GMP (Fig. 3A and C).

To independently assess the contribution of PdeH to c-di-GMP in cells, we employed liquid chromatography-tandem mass spectrometry (LC-MS/MS) to detect c-di-GMP levels in both PY79 wild-type and ΔpdeH strains grown to late log phase (OD_{600} of 1.0). A roughly 3-fold increase in c-di-GMP was observed by LC-MS/MS for the bulk population of ΔpdeH cells (see Fig. S5). We also attempted to quantify intracellular pGpG levels, but levels of pGpG were below the limit of detection for both wild-type and ΔpdeH cells (data not shown). A previous study quantified intracellular pGpG by LC-MS/MS in a *B. subtilis* mutant devoid of NrnA and NrnB, which are the two exoribonucleases thought to degrade pGpG in *Firmicutes* and other organisms that do not encode oligoribonuclease (Orn) (41). Similar to our current study, pGpG was undetectable in wild-type cells and could only be detected in the $\Delta\text{nrnA} \Delta\text{nrnB}$ double mutant. Therefore, we conclude that pGpG does not accrue to levels sufficiently high to interfere with the c-di-GMP *riboswitch-yfp* reporter.

Expression of the riboswitch reporter correlates with specific cell types. We next sought to correlate c-di-GMP levels with subpopulations of *B. subtilis*. We replaced

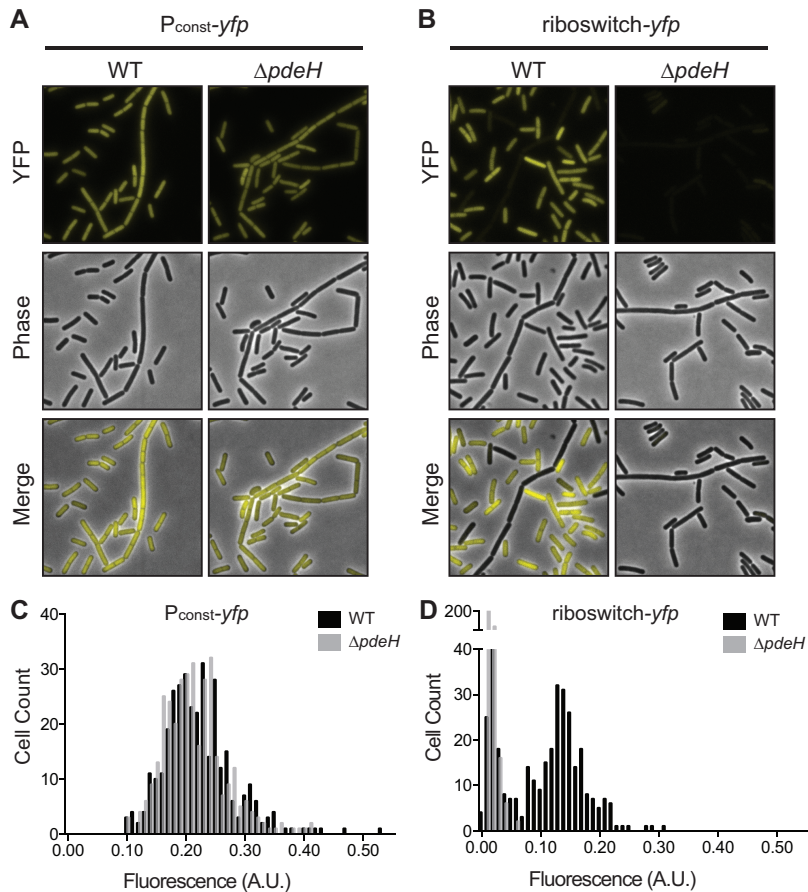


FIG 3 Deletion of *pdeH* results in increased c-di-GMP. (A and B) Representative microscopy images of *B. subtilis* PY79 wild-type (WT) or $\Delta pdeH$ cells expressing the constitutive P_{const} -yfp reporter (A) or the riboswitch reporter construct P_{const} -riboswitch-yfp (B). (C and D) Histograms of the quantification of fluorescence intensity per cell comparing *B. subtilis* PY79 WT or $\Delta pdeH$ expressing P_{const} -yfp (C) or P_{const} -riboswitch-yfp (D) ($n \approx 300$).

yfp with another gene encoding a red fluorescent protein, mCherry, and introduced the riboswitch reporter in strains that already harbored transcriptional *gfp* or *yfp* reporters that demarcate the most common *B. subtilis* cell types, including motile, matrix producing, or competent. When analyzed by fluorescence microscopy at late log phase (OD₆₀₀ of 1.0), a majority of cells exhibited high expression of a P_{hag} -*gfp* reporter (denoting motility) and high expression of the riboswitch-*mCherry* reporter (signifying low c-di-GMP). Of the cells that were transcriptionally active for motility gene expression, 89.8% also exhibited fluorescence from the riboswitch reporter. This suggests that c-di-GMP abundance is uniformly low in motile cells (Fig. 4A and D), and that a positive correlation exists between the genetic reporters. Low c-di-GMP levels were also observed in competent cells. While roughly 20% of cells in the population were competent, as indicated by a P_{comG} -yfp reporter, 79.7% of these cells exhibited high fluorescence from the c-di-GMP riboswitch-*mCherry* reporter (Fig. 4C and F). However, when we analyzed the riboswitch-*mCherry* reporter in the context of matrix-producing cells, as identified by a P_{tapA} -yfp reporter, we observed an anticorrelation between both reporters. Cells activated for producing extracellular matrix (as denoted by *tapA* gene expression) were observed mainly as long chains. Of these activated cells, 81.0% exhibited diminished fluorescence from the riboswitch-*mCherry* reporter (Fig. 4B and E). This suggests that c-di-GMP levels are high in matrix-producing cells. Conversely, cells that showed high fluorescence for the riboswitch-*mCherry* reporter were almost never activated for *tapA* expression. We did, however, observe a small population of cells that

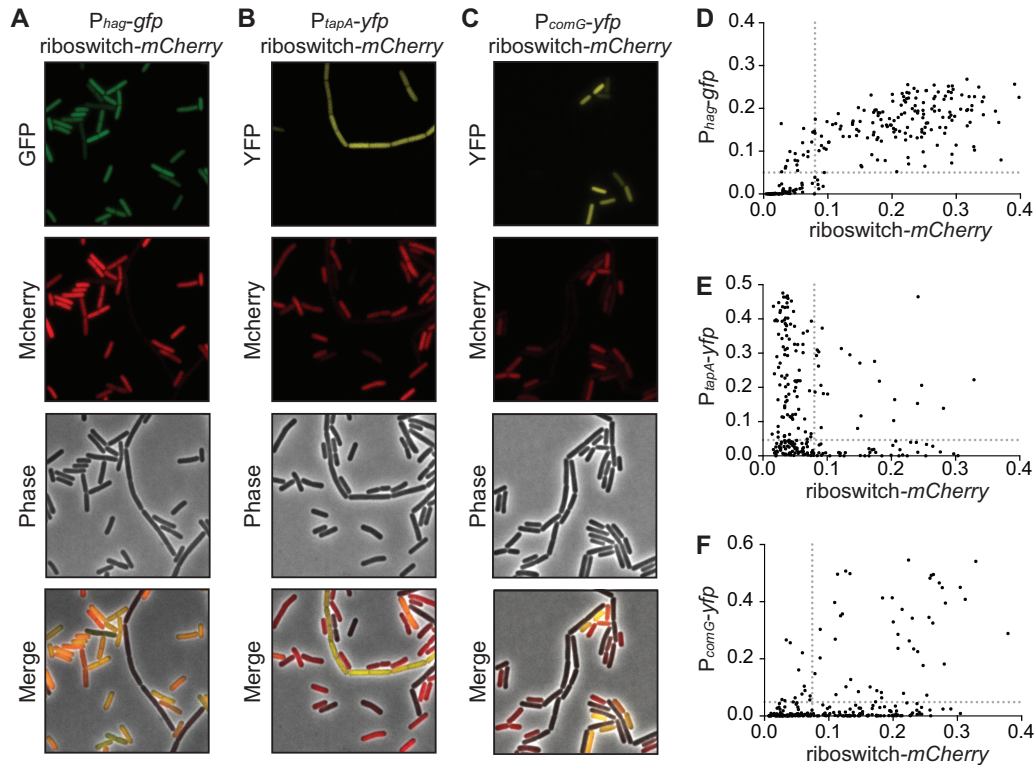


FIG 4 Expression of the riboswitch reporter varies nonrandomly among *B. subtilis* cell types. (A) Representative microscopy images of wild-type *B. subtilis* PY79 expressing the riboswitch reporter construct P_{const} -riboswitch-*mCherry* and the motility reporter $P_{\text{hag-gfp}}$. Statistical analyses show these reporters are significantly correlated ($P < 0.0001$). (B) Expression of P_{const} -riboswitch-*mCherry* and the biofilm reporter $P_{\text{tapA-yfp}}$ are anticorrelated ($P = 0.0039$). (C) Expression of P_{const} -riboswitch-*mCherry* and the competence reporter $P_{\text{comG-yfp}}$ are correlated ($P < 0.0001$). (D to F) Quantification of the fluorescence intensity per cell of P_{const} -riboswitch-*mCherry* compared to each cell type reporter in each construct ($n \approx 300$). Dotted lines represent the cutoff that divides fluorescent cells from nonfluorescent cells. Statistical significance was determined by chi-square analysis.

had c-di-GMP levels sufficiently high to shut off the riboswitch reporter but that had not activated the transcriptional reporter for biofilm formation ($P_{\text{tapA-yfp}}$). We speculate that these cells had not been activated yet for extracellular matrix production, which might suggest that c-di-GMP levels change prior to the activation of matrix production. Finally, we introduced the riboswitch-*yfp* reporter in a strain that also harbored a P_{const} -*mCherry* reporter and assessed c-di-GMP levels in cells that were activated for sporulation. Cells that progressed through spore development maintained expression of *mCherry* but were uniformly low in riboswitch-*yfp* expression, suggesting that c-di-GMP levels are high during endospore formation (Fig. 5).

An increase in c-di-GMP does not appear to change cell type identity. To see if the manipulation of intracellular c-di-GMP levels could influence the proportion of cells that became matrix producers (as well as motile or competent), we used the “high c-di-GMP” strain, $\Delta pdeH$ cells, and integrated both transcriptional reporters for c-di-GMP levels and cell type in this background. When cells were analyzed by fluorescence microscopy, we observed uniformly low levels of *mCherry* fluorescence, thereby confirming the elevated levels of c-di-GMP in the $\Delta pdeH$ strain (Fig. 6). Additionally, we observed no obvious consequence on cell fate at the transcriptional level. In many instances, however, c-di-GMP regulates phenotypic outputs at the posttranslational level (78–80). This is exemplified in *B. subtilis* motile cells, where the binding of MotI to c-di-GMP directs MotI to directly disengage MotA from the rotor of the flagellar apparatus (51). It is possible that c-di-GMP posttranslationally regulates YdaK as well, and this interaction activates production of the unknown exopolysaccharide (EPS). So, while the proportions of cells in each subtype were consistent between the wild type

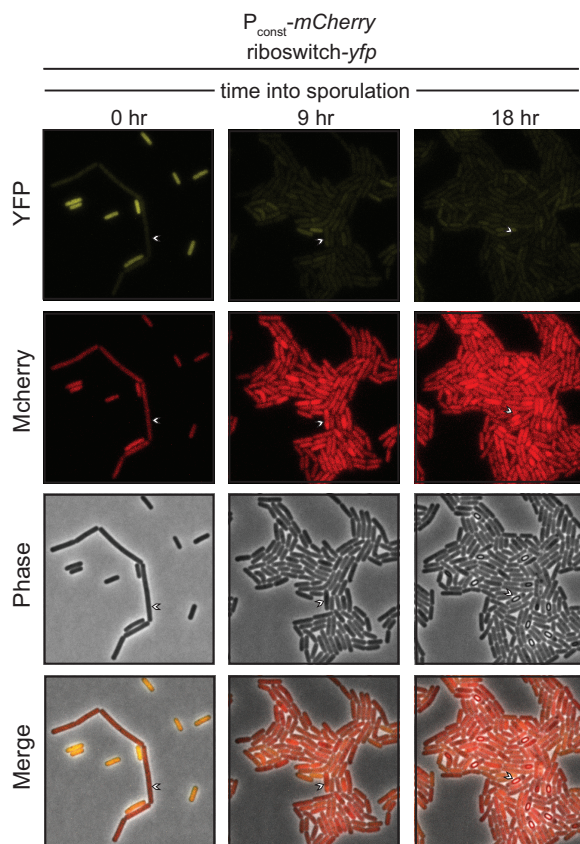


FIG 5 High c-di-GMP levels correlate with sporulation. Representative microscopy images of a time course through sporulation of the wild-type *B. subtilis* PY79 expressing the riboswitch reporter construct $P_{\text{const}}\text{-riboswitch-}yfp$ and the constitutive reporter $P_{\text{const}}\text{-}mCherry$.

and the $\Delta pdeH$ mutant, and the levels of activation for motility and matrix gene expression as measured by the reporters were the same (Fig. 6A, B, D, and E), it remains possible that c-di-GMP is a regulator of *B. subtilis*' differentiation at the posttranslational level. Interestingly, mild repression of the competence transcriptional reporter was observed in the $\Delta pdeH$ strain compared to that in the wild type (Fig. 6C and F).

***pdeH* expression is regulated transcriptionally.** In addition to the catalytic EAL domain that provides phosphodiesterase activity, some PDEs also contain sensory domains (e.g., PAS) that allow for posttranslational regulation of phosphodiesterase activity through activation by an extracellular signal. No such domain has been identified in *B. subtilis* PdeH. However, *pdeH* transcription was previously suggested to be repressed by Spo0A (49, 81) indicating that *pdeH* might be regulated transcriptionally. We therefore sought to ascertain if heterogeneous expression of the riboswitch reporter was due to heterogeneous expression of *pdeH* in each cellular subtype. We created a transcriptional fusion of the *pdeH* promoter to a reporter gene encoding Superfolder GFP ($P_{pdeH}\text{-}sf\text{-}gfp$) and ectopically integrated it into wild-type and $\Delta spo0A$ background strains. Mean fluorescence for the $\Delta spo0A$ background (0.162; 95% confidence interval [CI], 0.158 to 0.167) increased by roughly 2-fold compared to that of the wild type (0.086; 95% CI, 0.082 to 0.090) (Fig. 7A). This agrees with prior data showing Spo0A inhibition of *pdeH* expression (49). Furthermore, quantification of the $P_{pdeH}\text{-}sf\text{-}gfp$ reporter revealed a single peak, implying a normal distribution of *pdeH* expression across the population. While a $\Delta sigD$ mutation had little effect on mean fluorescence (0.075; 95% CI, 0.071 to 0.080) (Fig. 7C), a $\Delta sinR$ mutation (0.050; 95% CI, 0.046 to 0.054) led to a roughly 2-fold decrease in mean fluorescence (Fig. 7B). From this, we speculate that removal of SinR might lead to physiological changes that enhance the activation of Spo0A, thereby maximizing the Spo0A-mediated inhibition of *pdeH* transcription.

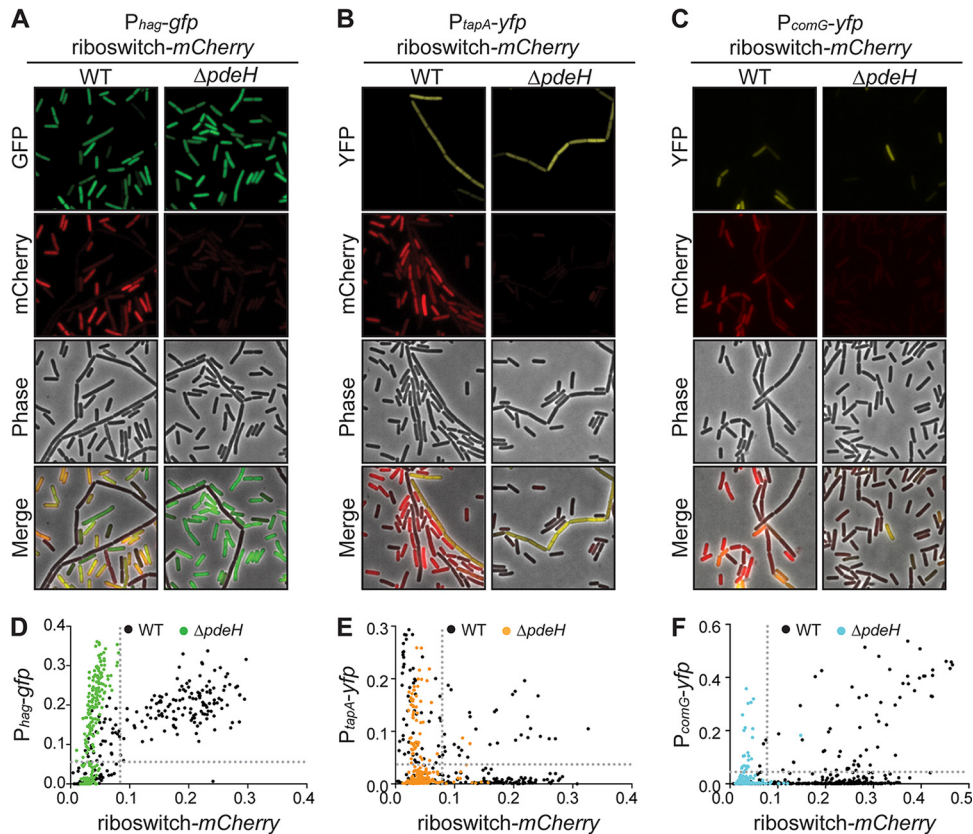


FIG 6 Deletion of *pdeH* does not change expression of each cell type reporter. (A to C) Representative microscopy images of *B. subtilis* PY79 wild-type (WT) or $\Delta pdeH$ expressing the riboswitch reporter construct P_{const} -riboswitch-mCherry and the motility reporter $P_{hag-gfp}$ (A), P_{const} -riboswitch-mCherry and the biofilm reporter $P_{tapA-yfp}$ (B), or P_{const} -riboswitch-mCherry and the competence reporter $P_{comG-yfp}$ (C). (D to F) Quantification of the fluorescence intensity per cell of P_{const} -riboswitch-mCherry compared to each cell type reporter, expressed in *B. subtilis* PY79 WT or $\Delta pdeH$ cells ($n \approx 300$).

Alternatively, SinR might activate expression of *pdeH*, directly or indirectly, through an unknown mechanism. We also quantified fluorescence from the riboswitch reporter in these genetic backgrounds to correlate *c*-di-GMP levels with activity from *pdeH*. Our data (Fig. 7A to C) predict that derepression of *pdeH* due to $\Delta spo0A$ should lead to lower intracellular *c*-di-GMP levels, exhibited by uniformly high fluorescence from the riboswitch reporter. Conversely, repression of *pdeH* due to $\Delta sinR$ should result in higher intracellular *c*-di-GMP levels and result in uniformly lower fluorescence from the riboswitch reporter. As predicted, our riboswitch reporter followed the hypothesized trends. A $\Delta spo0A$ mutation led to loss of a bimodal distribution of fluorescence, and the population exhibited similar fluorescence to the “low *c*-di-GMP” population of wild-type cells (Fig. 7D). Mean fluorescence in a $\Delta sinR$ mutant was uniformly low (0.023; 95% CI, 0.022 to 0.023), indicating high intracellular *c*-di-GMP levels (Fig. 7E). Unexpectedly, however, we observed uniformly low fluorescence from the riboswitch reporter in a $\Delta sigD$ background (0.029; 95% CI, 0.028 to 0.030). This suggests $\Delta sigD$ cells have an increase in intracellular *c*-di-GMP levels that cannot be explained by *pdeH* activity alone (Fig. 7C and F).

DISCUSSION

To study *c*-di-GMP abundance for single cells of *B. subtilis*, we employed a constitutively expressed *c*-di-GMP riboswitch fused to *yfp* as our biosensor. This riboswitch reporter revealed apparent differences in intracellular *c*-di-GMP levels among *B. subtilis* subpopulations. From these results, we suggest that *c*-di-GMP riboswitch-*yfp* reporters could be used in other bacteria—*Bacillales* in particular—for single-cell analyses of

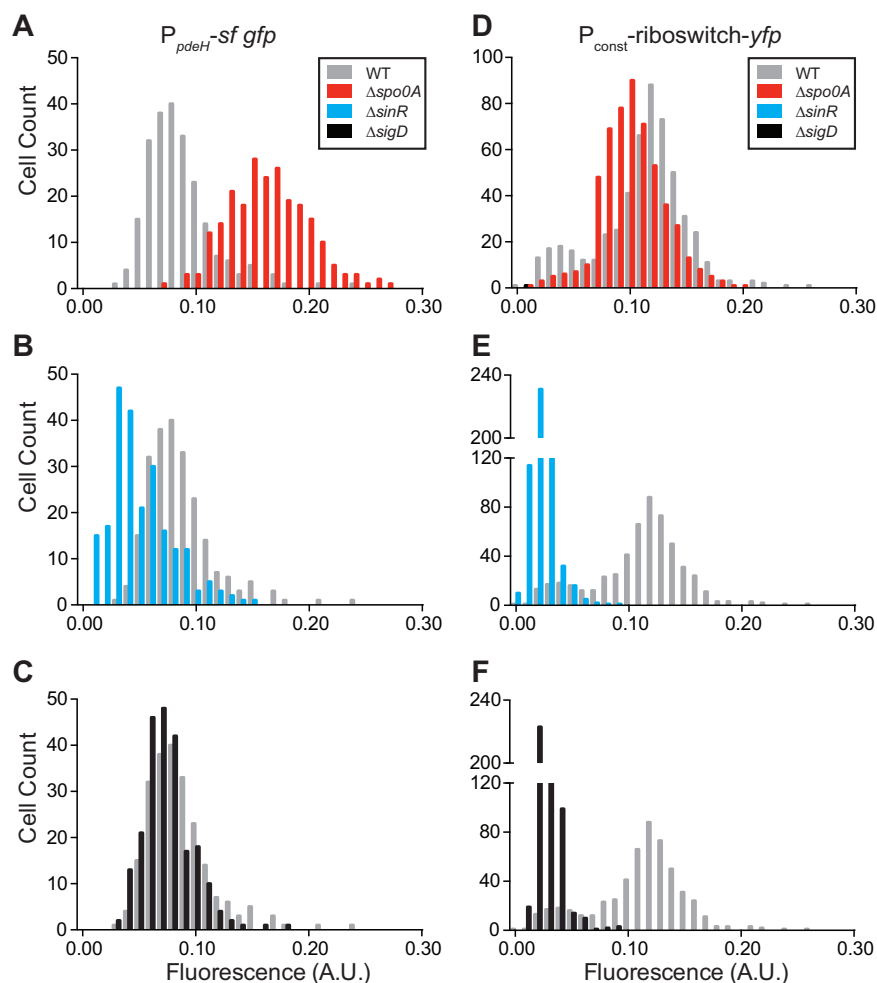


FIG 7 Deletion of global regulators affects *pdeH* expression and c-di-GMP levels. (A to C) Quantification of the fluorescence intensity per cell of P_{pdeH} -*sf-gfp* in *B. subtilis* PY79 wild-type (WT) and $\Delta spo0A$ (A), $\Delta sinR$ (B), and $\Delta sigD$ (C) genetic backgrounds ($n \approx 300$). (D to F) Quantification and comparison of the fluorescence intensity per cell of P_{const} -riboswitch-*mCherry* in *B. subtilis* PY79 wild-type (WT) and $\Delta spo0A$ (D), $\Delta sinR$ (E), and $\Delta sigD$ (F) genetic backgrounds ($n \approx 500$).

c-di-GMP abundance. These riboswitch-reporters may prove to be useful tools in elucidating the increasingly diverse c-di-GMP regulatory mechanisms used by Gram-positive bacteria. Studies on *Bacillus cereus* group organisms, *Clostridioides difficile*, and *Streptomyces coelicolor* have suggested a link between c-di-GMP metabolism and spore formation, which is a developmental program almost exclusive to the three genera (20). Interestingly, there is an ortholog of *B. subtilis* Ykul in the *B. cereus* bacterial group, which is named CdgJ. Overexpression of *Bacillus thuringiensis* *cdgJ* resulted in increased biofilm formation and earlier entry into sporulation (82). Conversely, no sporulation was observed in a *B. thuringiensis* *cdgJ* mutant. Given the high similarity in protein sequences between Ykul and CdgJ, it is therefore possible that Ykul also exhibits a similar role in *B. subtilis*, although it has yet to be explored. Sporulation is also affected by c-di-GMP in *S. coelicolor*. Overexpression of DGCs *cdgA* or *cdgB* or deletion of PDEs *rmdA* or *rmdB* led to an increase in intracellular c-di-GMP and inhibition of sporulation (83–85). Studies on *S. coelicolor* have also revealed the only c-di-GMP-sensing transcriptional regulator to date among Gram-positive organisms (BldD). Deletion of *bldD* accelerates sporulation (86), which is inhibited through overexpression of *cdgA* and *cdgB*, which are also direct targets of BldD. c-di-GMP-bound BldD also represses expression of antibiotic synthesis genes and sporulation genes during vegetative growth (83, 84). Together, these studies generally suggest a broadening role for

c-di-GMP signaling in Gram-positive bacteria. Our study herein extends this even further by showing that a c-di-GMP riboswitch is likely to control expression of a *B. licheniformis* lichenysin biosynthesis gene cluster. This observation suggests that in some bacteria, antibiotic synthesis is under the purview of c-di-GMP signaling. By extension, these data suggest that surfactin biosynthesis could also be subject to c-di-GMP regulation in other *Bacillus* species. Interestingly, a peptide essential for competence (ComS) is encoded within the surfactin operon *srfAA-AD* in *B. subtilis* (87). *srfAA-AD* is also regulated by the transcription factor ComA, which is ultimately activated by the quorum sensing molecule ComX (88–90). This regulatory arrangement allows *B. subtilis* to integrate a single signaling pathway into multiple adaptive processes. Prior studies have shown that surfactin-producing cells coexist with, but are phenotypically distinct from, cells that produce the extracellular matrix components (91). Therefore, our observation that c-di-GMP is low in competent cells implies that c-di-GMP should also be low for surfactin-producing cells. Indeed, the presence of the c-di-GMP riboswitch upstream of *B. licheniformis* *lchAA* operon indicates that lichenysin is produced only when c-di-GMP levels are low. ComA has been shown to also recognize the *B. licheniformis* *lchAA* promoter, further supporting a relationship between lichenysin and competence gene expression (92, 93). While a c-di-GMP-responsive riboswitch does not appear to be located in the leader of *B. subtilis* *srfAA*, it is possible that c-di-GMP could regulate this gene cluster by an as yet undiscovered mechanism. In summary, c-di-GMP influences a variety of cellular functions in Gram-positive bacteria, including sporulation and secondary metabolite production, and these discoveries may also foreshadow roles for c-di-GMP signaling during competence development in *B. subtilis*.

Other types of highly selective fluorescent c-di-GMP biosensors have also been developed in recent years. For example, a protein biosensor for c-di-GMP was previously developed from the *Salmonella enterica* serovar Typhimurium c-di-GMP binding protein YcgR (94, 95), which coupled binding of c-di-GMP to Förster resonance energy transfer (FRET). This genetically encoded biosensor protein was previously used for FRET-based microscopy of Gram-negative organisms such as *Caulobacter crescentus* and *Pseudomonas aeruginosa* (94, 96). However, FRET-based biosensors exhibit lower fluorescence intensities than individual fluorescent proteins, which can result in an overall narrowing of their dynamic range. Furthermore, many c-di-GMP protein receptors remain to be discovered in Gram-positive organisms for use in FRET microscopy. Riboswitch aptamers have also been used in prior studies as allosteric regulators of the conditionally fluorescent Spinach RNA for measuring c-di-GMP dynamics (64, 97). Yet, while they exhibit ideal performance characteristics *in vitro*, they require further optimization for routine usage in bacterial cells. For example, Spinach-based biosensors exhibit lower fluorescence than common fluorescent proteins. Also, significant amounts of DFHBI have to be added to cells for sufficient quantities of the chromophore to pass through the cell membrane. Some researchers have circumvented this limitation by tagging targeted RNAs with up to 64 spinach aptamers in tandem, thereby increasing the brightness of tagged RNA molecules (98); however, this approach is not ideal for biosensor purposes. In our experiments, we found that the constitutive YFP control in *B. subtilis* displayed more uniform fluorescence in a population of *B. subtilis* than the constitutive Spinach aptamer (see Fig. S3 in the supplemental material). Based on all of these considerations, we chose to pursue development of a riboswitch-*yfp* reporter fusion that could provide useful information on *B. subtilis* c-di-GMP abundance. The fluorescent proteins used in this study have also been used in multiple studies regarding *B. subtilis* cell differentiation (99, 100). They have been shown to exhibit a shortened half-life and therefore can be expected to report relative differences in gene expression; however, it remains to be determined whether these reporters are sufficient to measure rapid c-di-GMP dynamics.

Given the importance of *B. subtilis* as a model system for the study of bacterial development, it is essential that its c-di-GMP regulatory mechanisms be further elucidated. One of the most common assays to measure the impact of mutations, such as

those affecting c-di-GMP homeostasis, on biofilm formation is to visually assess the complexity of colony topology. While this can be qualitatively useful, it may not be representative of changes that occur at the cellular level. We take this to be due to the inherently heterogeneous composition of cell types within biofilm communities. Instead, the recent development of cell-type-specific fluorescent reporters allows investigators to examine features of *B. subtilis* subpopulations and their relationship to c-di-GMP effector proteins, DGCs, and PDEs. (51–53).

Herein, we coupled cell-type-specific transcriptional reporters with a c-di-GMP riboswitch-*yfp* reporter to ascertain whether c-di-GMP varies between *B. subtilis* subpopulations. Indeed, our single-cell microscopy shows definitively that intracellular c-di-GMP differs among *B. subtilis* cell types within a single population. Cells that have made the decision to become matrix producers maintain higher intracellular c-di-GMP levels than motile cells. This study also shows that the transition into endospore formation correlates with high c-di-GMP levels, while competent cells correlate with lower c-di-GMP levels. This trend was not previously examined in other studies. However, while our data demonstrate that c-di-GMP levels vary significantly between different cell types, it is unclear whether these changes in c-di-GMP abundance occur as a result of cellular decision making or whether they participate in initiating the choice of cell fate. Instead, we provide evidence that a general increase in c-di-GMP abundance is not likely to change the proportion of each cell type at the transcriptional level. While our data suggest that c-di-GMP metabolic enzymes act downstream of the master regulators that drive genetic regulation of cellular differentiation such as Spo0A and SinR (Fig. 7A and B), the molecular details of how c-di-GMP levels affect cellular differentiation require further exploration. Indeed, the general importance of c-di-GMP has yet to be fully ascertained for this organism. It is possible that the primary purpose of c-di-GMP signaling in this organism is to influence the motility apparatus. Alternatively, it is possible that effector targets for c-di-GMP-binding proteins have yet to be identified, as might be suggested by the recently discovered link to the *yda* exopolysaccharide pathway (52, 53). Overall, the abundance of c-di-GMP in a bulk ensemble of *B. subtilis* is likely averaged across the population, and we propose that single-cell analysis of c-di-GMP levels offers unique insight into how the abundance of this signaling molecule varies among cellular subpopulations. Analyses such as these are likely to offer new insight into c-di-GMP-regulated phenotypes, especially given that other biofilms also consist of multicellular communities.

MATERIALS AND METHODS

Transcription termination assay. PCR amplification was performed on the *B. licheniformis* *lchAA* leader sequence using primers that place it downstream of a constitutive promoter P_{const} and that ended 216 nucleotides (nt) downstream of the transcription start site (see Fig. S2 in the supplemental material). Transcription reactions were performed on the PCR-generated DNA template, resulting in a terminated (T) transcript of approximately 174 nt, or a 217-nt runoff (RO) transcript. These reaction mixtures comprised 5 μ M template, 250 μ M nucleoside triphosphates (NTPs), 1 \times *Escherichia coli* RNA polymerase reaction buffer (40 mM Tris-HCl [pH 7.5], 150 mM KCl, 10 mM MgCl₂, 1 mM dithiothreitol [DTT], 0.01% Triton X-100; NEB), 20 μ Ci [α -³²P]UTP, and 0.5 unit of *E. coli* RNA polymerase, holoenzyme (NEB). c-di-GMP was added to the runoff transcription reactions to final concentrations of 10 μ M, 100 μ M, and 1 mM. A 125-nt size marker was also transcribed. All reaction mixtures were incubated at 37°C for 2 h. Reaction results were resolved by 6% urea-denaturing polyacrylamide gel electrophoresis (PAGE).

Spinach activation assay. PCR was performed to amplify two aptamers: a tRNA^{Lys}-*lchAA*-Spinach2 template and a tRNA^{Lys}-Spinach 2 control template (Fig. S2). A forward annealing primer was used to introduce the T7 promoter sequence, resulting in 230-bp and 168-bp DNA templates, respectively. Transcription reactions were performed, and the products were purified and quantified as previously described (101). The *in vitro* Spinach2 fluorescence activation assay was modified from methods described previously (64). Briefly, the two RNA aptamers were each diluted to 2 μ M, added to an equal volume of 2 \times renaturation buffer (80 mM HEPES [pH 7.5], 250 mM KCl, 6 mM MgCl₂), heated to 70°C for 3 min, and cooled at room temperature for 5 min. To test the binding affinity of c-di-GMP for the *lchAA* aptamer, binding reactions were prepared for each RNA aptamer (40 mM HEPES [pH 7.5], 125 mM KCl, 3 mM MgCl₂, 100 nM RNA, 10 μ M DFHBI) and incubated in the dark at room temperature for 30 min. Stocks of c-di-GMP or pGpG were prepared and added to the binding reaction mixture every 30 min to achieve the final concentrations that are graphed in the figures. Ligands were added until saturation of fluorescence was reached. A Quantus fluorometer (Promega) was used to excite the reaction mixture at 495 nm every 30 min after incubation with increasing amounts of c-di-GMP and pGpG. Binding to

c-di-AMP was also assessed. The experiment was replicated twice, and the background fluorescence of DFHBI alone was subtracted from all data points.

Culture conditions and construction of *B. subtilis* strains. All *B. subtilis* strains in this study were derived from PY79 (unless otherwise noted) and are listed in Table S1. Assessment of *B. subtilis* 3610 was performed with the use of DS7187, a competent 3610 $\Delta comI$ mutant (provided by D. Kearns, Indiana University) (102). A table of the primers and plasmids used is available upon request. Strains were grown at 37°C on lysogeny broth (LB) plates supplemented with 1.5% Bacto agar and, when appropriate, with final concentrations of the following antibiotics: 5 $\mu\text{g/ml}$ chloramphenicol, and 1 $\mu\text{g/ml}$ erythromycin added alongside 25 $\mu\text{g/ml}$ lincomycin. Integration at the *amyE* locus was performed with plasmids derived from pJG019 (GenBank accession no. [KX499653.1](https://doi.org/10.1007/s12220-010-9653-1)) or pVMZ006, both derivatives of pDG1662 (BGSC). To construct pRSL_F4, the *IchAA* leader sequence was synthesized (GenScript) and subcloned into the HindIII restriction site of pJG019. For construction of the P_{pdeH} -*sf gfp* reporter, the constitutive promoter of pVMZ006 was replaced with the *pdeH* promoter sequence that was described previously (49). The sequence of *sf gfp* was amplified from pJ204:102624 (DNA2.0, Inc.) and was used to replace the *yfp* sequence of pVMZ006 via Gibson assembly (103). The fluorescent transcriptional cell type reporters P_{hag} -*gfp*, P_{tapA} -*yfp*, and P_{comG} -*yfp* that were used in this study were described previously (4, 5, 104, 105). To construct the plasmids harboring these reporters, promoter sequences of *tapA* and *comG* were amplified from *B. subtilis* and used to replace the constitutive promoter upstream of *yfp* in pVMZ006 by Gibson assembly (103). The *amyE::P_{hag}-gfp* fusion from DS4432 (provided by D. Kearns, Indiana University) was introduced into the PY79 chromosome through double homologous recombination of competent cells. Plasmids derived from pDG1664 (BGSC) were used for integration at the *thrC* locus. To make markerless deletions of $\Delta pdeH$, $\Delta spo0A$, $\Delta sinR$, and $\Delta sigD$, strains harboring an erythromycin resistance cassette inserted in loci BSU31740 (*pdeH*), BSU24220 (*spo0A*), BSU24610 (*sinR*), and BSU16470 (*sigD*) were obtained from the BKE collection (BGSC). Markerless deletions were created through transformation with pDR244 (BGSC), as previously described (106). Removal of the erythromycin-resistance cassette was verified by Sanger sequencing. Transformation of PY79 was performed using a previously described protocol (107).

Fluorescence microscopy and quantification. Single colonies were used to inoculate liquid minimal salts glycerol glutamate (MSgg) medium (75) and grown at 37°C with shaking overnight. The following morning, cultures of each strain were inoculated 1:50 in fresh medium and grown at 37°C with shaking until reaching an optical density at 600 nm (OD_{600}) of 1.0. Aliquots of these cultures were placed on 1.5% low-melting-point agarose MSgg pads and allowed to dry for 10 min. Agarose pads were inverted on a glass bottom dish (Willco Wells). Cells were imaged at room temperature using a Zeiss Axio-Observer Z1 inverted fluorescence microscope equipped with a Rolera EM-C₂ electron-multiplying charge-coupled (EMCC) camera, enclosed within a temperature-controlled environmental chamber. Fluorescence intensity per cell was quantified using Oufiti analysis software (108). Images were analyzed and adjusted with Fiji software (109).

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <https://doi.org/10.1128/JB.00247-19>.

SUPPLEMENTAL FILE 1, PDF file, 0.8 MB.

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