c-di-GMP Inhibits Early Sporulation in Clostridioides difficile

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ABSTRACT The formation of dormant spores is essential for the anaerobic pathogen Clostridioides difficile to survive outside the host gastrointestinal tract. The regulatory pathways and environmental signals that initiate C. difficile spore formation within the host are not well understood. One second-messenger signaling molecule, cyclic diguanylate (c-di-GMP), modulates several physiological processes important for C. difficile pathogenesis and colonization, but the impact of c-di-GMP on sporulation is unknown. In this study, we investigated the contribution of c-di-GMP to C. difficile sporulation. The overexpression of a gene encoding a diguanylate cyclase, dccA, decreased the sporulation frequency and early sporulation gene transcription in both the epidemic R20291 and historical 630Δerm strains. The expression of a dccA allele encoding a catalytically inactive DccA that is unable to synthesize c-di-GMP no longer inhibited sporulation, indicating that the accumulation of intracellular c-di-GMP reduces C. difficile sporulation. A null mutation in dccA slightly increased sporulation in R20291 and slightly decreased sporulation in 630Δerm, suggesting that DccA contributes to the intracellular pool of c-di-GMP in a strain-dependent manner. However, these data were highly variable, underscoring the complex regulation involved in modulating intracellular c-di-GMP concentrations. Finally, the overexpression of dccA in known sporulation mutants revealed that c-di-GMP is likely signaling through an unidentified regulatory pathway to control early sporulation events in C. difficile. c-di-GMP-dependent regulation of C. difficile sporulation may represent an unexplored avenue of potential environmental and intracellular signaling that contributes to the complex regulation of sporulation initiation.

IMPORTANCE Many bacterial organisms utilize the small signaling molecule cyclic diguanylate (c-di-GMP) to regulate important physiological processes, including motility, toxin production, biofilm formation, and colonization. c-di-GMP inhibits motility and toxin production and promotes biofilm formation and colonization in the anaerobic, gastrointestinal pathogen Clostridioides difficile. However, the impact of c-di-GMP on C. difficile spore formation, a critical step in this pathogen’s life cycle, is unknown. Here, we demonstrate that c-di-GMP negatively impacts sporulation in two clinically relevant C. difficile strains, the epidemic strain R20291 and the historical strain 630Δerm. The pathway through which c-di-GMP controls sporulation was investigated, and our results suggest that c-di-GMP is likely signaling through an unidentified regulatory pathway to control C. difficile sporulation. This work implicates c-di-GMP metabolism as a mechanism to integrate environmental and intracellular cues through c-di-GMP levels to influence C. difficile sporulation.

KEYWORDS Clostridioides difficile, Clostridium difficile, sporulation, spore, cyclic diguanylate, c-di-GMP, anaerobe, cyclic diguanylate synthase

Nucleotide second messengers, such as the nearly ubiquitous cyclic diguanylate (c-di-GMP), serve as central intracellular signaling molecules in bacteria. c-di-GMP promotes the switch between a planktonic, motile stage and a sessile, surface-associated
We show that the overexpression of ability and/or other environmental conditions to the decision to initiate sporulation. (32), by the Opp and App oligopeptide permeases affects the timing of sporulation, as gets for CodY and CcpA regulation, but the molecular mechanisms are not delineated increased sporulation frequencies (30, 31). Some sporulation genes serve as direct tar-

Onset of sporulation in C. difficile is an obligate anaerobe and relies on the formation of a dormant spore for long-term persistence outside the host and transmission to new hosts. Spore formation in all endospore-forming bacteria, including C. difficile, is initiated by the highly con-
served transcriptional regulator Spo0A (21, 22). Spo0A activity is tightly controlled by phosphorylation through a large regulatory network of kinases, phosphatases, and addi-
tional regulators (23, 24). Once phosphorylated, Spo0A→P activates the expression of early sporulation genes, triggering sporulation (25; M. A. DiCandia and S. M. McBride, unpublished data). However, many of the regulatory proteins and pathways that control early sporulation events in the model organism Bacillus subtilis are not conserved in C. difficile (26, 27). Although recent progress has uncovered several important sporulation regulatory factors in C. difficile (reviewed in reference 28), the environmental cues and regulatory pathways that control Spo0A activation are largely unknown.

Environmental conditions and nutrient availability likely trigger C. difficile spore formation within the host gastrointestinal tract. Two global transcriptional regulators, CodY and CcpA, control sporulation initiation in C. difficile in response to nutrient avail-

ability. CodY represses target gene expression when GTP and branched-chain amino acids are abundant, and CcpA activates or represses target gene transcription based on carbohydrate availability (29, 30). Mutations in either CodY or CcpA result in increased sporulation frequencies (30, 31). Some sporulation genes serve as direct targets for CodY and CcpA regulation, but the molecular mechanisms are not delineated (29–31). Additionally, the uptake of peptides, a critical nutrient source for C. difficile (32), by the Opp and App oligopeptide permeases affects the timing of sporulation, as opp and app inactivation significantly increases sporulation frequencies (33). It is reason-
able to consider that other global signaling systems in C. difficile link nutrient avail-

ability and/or other environmental conditions to the decision to initiate sporulation.

In this study, we examined the impact that c-di-GMP has on C. difficile sporulation. We show that the overexpression of dccA, a gene encoding a DGC, resulted in decreased sporulation in two important C. difficile strains. The conserved catalytic motif GGDEF was repons;ed for DccA-dependent inhibition of C. difficile spore formation, indicating that c-
di-GMP metabolic activity is responsible for this phenotype. Finally, we provide evidence that c-di-GMP does not depend on signaling through several known sporulation factors, suggesting that c-di-GMP influences sporulation through an unidentified pathway.

RESULTS

Overexpression of a diguanylate cyclase reduces C. difficile sporulation frequency. As c-di-GMP affects many physiological processes in C. difficile, we hypothesized that C. difficile sporulation is also influenced by c-di-GMP. To test this hypothesis, dccA, which encodes a DGC in C. difficile (10, 13), was overexpressed on a multicopy plasmid using lifestyle and controls virulence factor production in numerous pathogenic and nonpa-
thogenic bacteria. c-di-GMP is synthesized from GTP by diguanylate cyclases (DGCs) (syn-
thases), which contain the conserved catalytic GGDEF motif (1). Phosphodiesterases (PDEs) (hydrolases) containing either the EAL or HD-GYP domain degrade c-di-GMP to pGpG or GMP, respectively (2–5). Often, DGC and PDE proteins contain additional sen-
sory or regulatory domains that potentially control enzymatic activity, suggesting that environmental and bacterial cues influence the intracellular concentration of c-di-GMP (6). Most Gram-negative bacteria encode numerous DGC and PDE proteins, resulting in complex c-di-GMP metabolic pathways, while Gram-positive bacteria often contain more modest numbers of DGC and PDE proteins (7–9). However, the gastrointestinal pathogen Clostridioides difficile encodes many c-di-GMP metabolic proteins; 37 genes encoding GGDEF and/or EAL domains were identified in the historical 630 strain (10, 11). Many of the C. difficile c-di-GMP metabolic proteins have been demonstrated to be enzymatically active (10, 12), suggesting that the regulation of c-di-GMP metabolism in C. difficile is physiologically important and tightly controlled. High intracellular concentrations of c-di-GMP have been demonstrated to inhibit C. difficile motility and toxin production while promoting cell aggregation, biofilm formation, and colonization (13–20). c-di-GMP metabolic proteins have been demonstrated to be enzymatically active (10, 12), suggesting that the regulation of c-di-GMP metabolism in C. difficile is physiologically important and tightly controlled. High intracellular concentrations of c-di-GMP have been demonstrated to inhibit C. difficile motility and toxin production while promoting cell aggregation, biofilm formation, and colonization (13–20).
the nisin-inducible cpr promoter (13, 33, 34) in two different C. difficile backgrounds. We included R20291, which is a clinically prevalent epidemic strain, and 630Δerm, which is a spontaneous erythromycin-sensitive derivative of 630, a clinical isolate that has served as a long-term laboratory model strain (35, 36). Of note, the amino acid sequences of DccA from R20291 and 630Δerm are 100% identical. To assess sporulation frequency, we performed ethanol-resistant sporulation assays in these strains after 24 h of growth (H24) on 70:30 sporulation agar.

When a plasmid copy of dccA (pDccA) was overexpressed from the nisin-inducible promoter in the R20291 background, the sporulation frequency significantly decreased from 33.7% in the absence of nisin to 19% in the presence of 0.5 μg/ml nisin (Fig. 1A), suggesting that the overexpression of dccA and, presumably, high intracellular levels of c-di-GMP inhibit C. difficile sporulation. To assess whether the decrease in the sporulation frequency was due to the production of c-di-GMP by DccA, we overexpressed a dccA allele encoding a mutated GGDEF domain (AADEF) (13). Here, even at the highest expression level of dccA (0.5 μg/ml nisin), the sporulation efficiency remained unaffected, indicating that the DccA-dependent reduction in the sporulation frequency is due to DccA’s diguanylate cyclase activity. We also visualized sporulation in these strains using phase-contrast microscopy, in which spores appear phase bright and vegetative cells are phase-dark rods.
When dccA was overexpressed in R20291, not only were fewer spores visible, but this strain also formed long chains (Fig. 1B; discussed below). The reduced sporulation frequency and cell morphology phenotypes are dependent on increased c-di-GMP concentrations as these effects were not seen in the R20291 strain overexpressing pDccAmut (data not shown).

When dccA was overexpressed in the 630Δerm background, we observed a 4-fold decrease in the sporulation efficiency (29.7% in the absence of nisin to 8.5% with 0.5 mg/ml nisin) (Fig. 1C). This reduction in sporulation frequency in the 630Δerm pDccA strain was dose dependent, with the lowest sporulation frequency occurring at the highest expression level of dccA (0.5 μg/ml nisin). Again, the overexpression of the dccAmut allele resulted in wild-type (WT) levels of sporulation, indicating that the ability of DccA to repress sporulation relies on a functional GGDEF domain and its diguanylate cyclase activity. Similar to R20291, the overexpression of dccA in the 630Δerm background resulted in fewer spores and a change in cell morphology when observed by phase-contrast microscopy, and these phenotypes were also dependent on a functional DccA GGDEF domain (Fig. 1D and data not shown).

As noted above, the overexpression of dccA resulted in dramatic cell morphology changes in R20291 and 630Δerm that are dependent on increased c-di-GMP concentrations as these effects were not seen in the R20291 strain overexpressing pDccAmut (data not shown).

Overexpression of a cyclic diguanylate decreases sporulation-specific gene expression. To ensure that dccA and dccAmut transcription are activated in a dose-dependent manner with increasing concentrations of nisin, we measured dccA transcript levels using quantitative reverse transcription-PCR (qRT-PCR). Cells were harvested
after 12 h of growth on 70:30 sporulation agar, which marks early stationary phase and the onset of sporulation (39). As expected, dccA and dccA\textsuperscript{mut} transcript levels were increased ~4- to 6-fold in both R20291 and 630\textDeltaerm in the presence of 0.5 \(\mu\)g/ml nisin (Fig. 3A and B), showing that dccA and dccA\textsuperscript{mut} expression levels are consistent in both backgrounds. To better understand the effect of c-di-GMP on early sporulation events in \textit{C. difficile}, we measured the steady-state transcript levels of an early sporulation-specific gene, sigE (spoIIG), using qRT-PCR. The transcription of sigE is dependent on active, phosphorylated Spo0A (40). The relative expression levels of sigE in both R20291 pDccA and 630\textDeltaerm pDccA grown on 0.5 \(\mu\)g/ml nisin were decreased ~2-fold compared to the levels in their respective parent strains (Fig. 3C and D). The decreased sigE transcript levels in R20291 pDccA were dependent upon a functional GGDEF domain.

![Diagram](image-url)
TABLE 1 Effect of dccA overexpression on sporulation-specific gene expression in R20291 and 630Δerm

| Transcript | Strain | Mean fold change ± SD<sup>c</sup> |
|------------|--------|---------------------------------|
|            | pMC211 | pDccA | pDccA<sub>mut</sub> |
| spo0A      | R20291 | 0.91 ± 0.12 | 0.96 ± 0.27 | 0.77 ± 0.11 |
|            | 630Δerm| 0.79 ± 0.07 | 0.96 ± 0.16 | ND |
| murG       | R20291 | 0.85 ± 0.08 | 0.72 ± 0.08 | 0.79 ± 0.06 |
| (Spo0A dependent) | 630Δerm| 1.19 ± 0.15 | **0.60 ± 0.05** | ND |
| sigF       | R20291 | 0.97 ± 0.20 | 1.15 ± 0.38 | 0.95 ± 0.23 |
|            | 630Δerm| 0.74 ± 0.10 | 0.47 ± 0.11 | ND |
| gpr        | R20291 | 0.82 ± 0.10 | 0.6 ± 0.17 | 0.9 ± 0.10 |
| (SigF dependent) | 630Δerm| 1.16 ± 0.19 | **0.44 ± 0.15** | ND |
| spoIID     | R20291 | 0.73 ± 0.14 | 0.48 ± 0.08 | 0.67 ± 0.13 |
| (SigE dependent) | 630Δerm| 1.32 ± 0.13 | **0.72 ± 0.06** | ND |

<sup>a</sup>All mean fold change values are relative to the respective parent strain containing pMC211 grown in the presence of no nisin.
<sup>b</sup>Strains were harvested at H12 from 70:30 sporulation agar supplemented with 2 μg/ml thiamphenicol and 0.5 μg/ml nisin.
<sup>c</sup>Boldface type indicates a P value of ≤0.05 by Student’s t test (630Δerm background). There was no statistically significant data in the R20291 background as determined by one-way ANOVA followed by Dunnett’s multiple-comparison test (R20291 background). ND, not determined.

As sigE transcript levels were unchanged when dccA<sub>mut</sub> was overexpressed in R20291 (Fig. 3C).

To further assess how c-di-GMP impacts early sporulation events, we measured the transcript levels of additional early-sporulation-specific genes, including spo0A, murG (a Spo0A-dependent gene), sigF (encodes the early sporulation-specific sigma factor that is expressed in the forespore compartment), gpr (a SigF-dependent gene), and spoIID (a SigE-dependent gene). The transcript levels of early sporulation genes were minimally affected in the R20291 background; however, with the exception of spo0A, there was a significant decrease in early sporulation gene expression when dccA was overexpressed in the 630Δerm background (Table 1). These results may reflect the stronger impact on sporulation that dccA overexpression has on 630Δerm than on R20291. Mirroring spo0A transcript levels in R20291 and 630Δerm, Spo0A protein levels were unchanged regardless of dccA overexpression (Fig. 3E). The lack of changes in spo0A transcript and protein levels is expected given that Spo0A activity is controlled by posttranslational phosphorylation. Furthermore, Spo0A autoregulation has not been observed in other C. difficile sporulation mutants, including the hypersporulating opp app and oligosporogenous rstA mutants (33, 39), despite affecting Spo0A activity and Spo0A-dependent gene expression. These data indicate that the overexpression of dccA reduces early sporulation gene expression and confirm that the increased production of c-di-GMP is responsible for reduced sporulation in C. difficile.

**Overexpression of dccA increases c-di-GMP-dependent gene expression in a dose-dependent manner on 70:30 sporulation agar.** We previously utilized high-performance liquid chromatography–tandem mass spectrometry (HPLC-MS/MS) to measure the intracellular concentration of c-di-GMP when dccA is overexpressed from the nisin-inducible promoter (13). To assess the relative increase in intracellular c-di-GMP with dccA induction during growth on 70:30 sporulation agar, we employed the previously described reporter controlled by the regulatory region of the pilA1 locus: the pilA1 promoter (P<sub>pilA1</sub>) and the 5’ untranslated region (UTR) containing a c-di-GMP riboswitch (41). c-di-GMP directly and positively regulates pilA transcription via the c-di-GMP-responsive riboswitch located in the pilA 5’ UTR (16). This P<sub>pilA1</sub>-UTR-gusA reporter was introduced into 630Δerm with chromosomal nisin-inducible dccA (41). As a control, we also utilized a P<sub>pilA1</sub>-UTR<sup>700C</sup>-gusA reporter where a single nucleotide substitution renders the riboswitch unresponsive.
We measured $P_{\text{pilA1}}$-UTR-$\text{gusA}$ reporter activity after growth on 70:30 sporulation agar in the presence of no nisin or 0.1 mg/ml or 0.5 mg/ml nisin at H12 and H15. These time points represent early stationary phase, at the point where the sporulation regulatory cascade is initiated and sporulation-specific gene expression is active. Without induction, basal expression from the chromosomally encoded $P_{\text{cpr-dccA}}$ construct resulted in a slight increase (1.2-fold) in $\beta$-glucuronidase activity compared to that of the parent strain at both time points (Fig. 4A). We observed 1.4-fold and 1.6-fold increases in $\beta$-glucuronidase activity in the presence of 0.1 mg/ml and 0.5 mg/ml nisin, respectively, compared to the parental control. As expected, the c-di-GMP-blind $P_{\text{pilA1}}$-UTR-$\text{gusA}$ reporter exhibited significantly reduced and constitutive activity under all tested conditions (Fig. 4A).

Because $\text{dccA}$ is overexpressed from the chromosome in these reporter assays and not from a plasmid as we did in our previous experiments, we assessed the impact on sporulation frequency when $\text{dccA}$ expression was induced from the chromosome. The sporulation frequency of $630\Delta\text{erm}::P_{\text{cprA-dccA}}$ grown on 70:30 sporulation agar plates supplemented with 0.5 $\mu$g/ml nisin was 1.44-fold decreased compared to the $630\Delta\text{erm}$ parent (Fig. 4B). As anticipated, the decrease in the sporulation efficiency was muted compared to $\text{dccA}$ overexpression on the plasmid, likely due to differences in copy numbers. However, increasing the intracellular c-di-GMP concentration through $\text{dccA}$ overexpression impacts both c-di-GMP-specific gene regulation and sporulation frequency. Altogether, these data confirm that c-di-GMP levels are modestly induced to physiologically relevant concentrations that affect c-di-GMP-dependent physiological processes under these conditions.

**Deletion of $\text{dccA}$ results in variable sporulation frequencies in R20291 and 630Aerm.** Sporulation may be impacted by one or a subset of c-di-GMP metabolic enzymes. *C. difficile* 630 encodes 37 proteins containing GGDEF and/or EAL domains, and R20291 encodes 31 (10, 11, 35). The overexpression of $\text{dccA}$ bypasses the endogenous control of c-di-GMP. To better understand the c-di-GMP regulatory mechanism, we next asked whether a null mutation in $\text{dccA}$ alone affects the *C. difficile* sporulation frequency. To test this hypothesis, we employed the pseudo-suicide vectors pMSR and pMSR0 (tailored for the 630 and R20291 backgrounds, respectively), which take advantage of allelic exchange and an inducible *C. difficile* toxin-antitoxin system to create a markerless gene deletion (42).

The R20291 $\Delta\text{dccA}$ mutant produced slightly more spores than R20291 (42.3% in R20291 $\Delta\text{dccA}$ versus 31.7% in the isogenic parent) (Fig. 5A). However, the sporulation...
frequencies were highly variable, and the differences between strains were not statistically significant. The R20291 ΔdccA mutant was complemented by integrating the dccA gene into the chromosome using the conjugative transposon Tn916. dccA is the second gene in a two-gene operon in R20291 and 630Δerm, and the promoter of the upstream gene, CD1421, was used to drive dccA transcription in the complementation construct. The sporulation frequency of R20291 ΔdccA Tn916::P_CD1421-dccA was reduced (34.7%) compared to that of the dccA mutant, but again, the data were variable and not statistically significant.

The 630Δerm ΔdccA mutant exhibited a slightly reduced sporulation frequency compared to the 630Δerm parent (19.1% in the dccA mutant versus 25.6% in the parent strain) (Fig. 5B), an opposite trend compared to that of the dccA mutant in the R20291 background, the sporulation frequencies were variable and did not achieve statistical significance. Altogether, these data suggest that c-di-GMP synthesis by DccA does not greatly and/or consistently contribute to sporulation initiation under the conditions tested or that other c-di-GMP metabolic enzymes are redundant with or compensate for the loss of DccA.

c-di-GMP likely does not signal through known C. difficile sporulation factors.

To attempt to identify the regulatory pathway(s) through which c-di-GMP influences sporulation in C. difficile, we overexpressed dccA in several well-studied 630Δerm sporulation mutants and performed ethanol-resistant sporulation assays after 24 h of growth on 70:30 sporulation agar. We included the parent 630Δerm strain overexpressing dccA in the absence and presence of 0.5 μg/ml nisin as a reference in these experiments (Fig. 6A).

First, we assessed the effect of dccA overexpression on 630Δerm codY and 630Δerm opp app mutants. CodY is a global transcriptional regulator that binds to target DNA at high intracellular concentrations of the effectors GTP and branched-chain amino acids (BCAAs) (43). The loss of GTP and BCAA binding changes the conformation of CodY, the differential expression of metabolic pathways, and other physiological processes, including toxin production and sporulation (29, 31, 44, 45). The opp and app operons each encode oligopeptide permeases that import small, heterogeneous peptides into the cell (46, 47). The inactivation of these permeases in C. difficile results in significantly increased sporulation, suggesting that limited nutrient uptake triggers sporulation

![Graph showing sporulation efficiency](image-url)
The regulatory pathways and molecular mechanisms by which CodY, Opp, and App affect sporulation are not fully understood, although null mutations in these loci affect sporulation timing and result in increased sporulation frequencies (31, 33). The sporulation frequencies of the \textit{codY} mutant and the \textit{opp app} double mutant decreased when \textit{dccA} was overexpressed using 0.5 \( \mu \text{g/ml} \) nisin (2-fold and 3.7-fold, respectively, compared to each strain’s vector controls grown in 0.5 \( \mu \text{g/ml} \) nisin) (Fig. 6B and C). These results indicate that c-di-GMP does not require CodY or the Opp and App oligopeptide permeases to inhibit \textit{C. difficile} sporulation.

Next, we ascertained whether RstA or the phosphotransfer proteins CD1492 and CD2492 are part of the regulatory pathway that c-di-GMP employs to control sporulation. RstA is a multifunctional protein that serves as a transcriptional regulator and, through a separate domain, positively influences \textit{C. difficile} sporulation initiation via an unknown mechanism (39). The overexpression of \textit{dccA} in the \textit{rstA} background resulted in an 13-fold-decreased sporulation frequency compared to that of the \textit{rstA} mutant containing the vector control (Fig. 6D), indicating that RstA is not necessary for c-di-GMP-dependent inhibition of sporulation. CD1492 and CD2492 are predicted histidine kinases that function as phosphotransfer proteins to repress sporulation and are
hypothesized to directly impact Spo0A phosphorylation (28, 48). A CD1492 CD2492 double mutant exhibited significantly increased sporulation compared to the 630Δerm parent (Fig. 6A and E), and the overexpression of dccA in the CD1492 CD2492 background reduced the sporulation frequency ~3-fold (Fig. 5E), demonstrating that CD1492 and CD2492 are not a required part of the c-di-GMP signaling pathway.

We also asked whether c-di-GMP signals through SigD to control C. difficile sporulation. SigD is the flagellar alternative sigma factor that is required for both motility and efficient toxin production in C. difficile (14, 39, 49). Although a null sigD mutation does not affect sporulation under these conditions (comparing the 630Δerm and 630Δerm sigD vector control strains) (Fig. 6A and F) (39, 49), we chose to investigate the sigD mutant because c-di-GMP directly represses sigD transcription, inhibiting C. difficile motility and toxin production (13, 15, 50). The sporulation frequency was reduced only 1.9-fold when dccA was overexpressed in the sigD background, and the difference was not statistically significant (Fig. 6F). Furthermore, in the 630E background, where the phase-variable switch that controls the transcription of the flagellar operon is locked off, resulting in low sigD expression levels (51–53), the overexpression of dccA also significantly decreased sporulation by ~20-fold (Fig. 6G). The DccA-dependent effect was not as dramatic in the 630Δerm sigD background as in the other mutant backgrounds tested; however, these data altogether suggest that c-di-GMP does not primarily influence sporulation through SigD.

Finally, we tested whether c-di-GMP affects sporulation frequency through the catabolite control protein CcpA. As a transcriptional regulator, CcpA responds to glycolytic carbohydrate availability to regulate carbon and nitrogen metabolism as well as other physiological processes, including sporulation and toxin production (30, 54). The sporulation frequency was again significantly decreased by ~20-fold when dccA was overexpressed in the 630E ccpA mutant (Fig. 6H), indicating that CcpA is not involved in c-di-GMP signaling to control sporulation initiation. Altogether, these data indicate that c-di-GMP does not significantly impact C. difficile sporulation through these known regulatory factors under the conditions tested.

DISCUSSION

In this work, we set out to determine the impact of the bacterial second messenger c-di-GMP on C. difficile sporulation. We found that the overexpression of dccA, encoding a DGC that synthesizes high intracellular levels of c-di-GMP when overexpressed (13), resulted in significant decreases in early sporulation gene expression and spore formation in the epidemic R20291 and historical 630Δerm strains. The conserved catalytic GGDEF motif was required for DccA-dependent inhibition of sporulation, indicating that the diguanylate cyclase synthase activity of DccA is responsible for decreased sporulation. Consistent with this result, sporulation was inhibited in a dose-dependent manner, as higher transcript levels of dccA coincided with fewer detected transcripts of sigE, which encodes an early sporulation-specific sigma factor.

Because DccA overexpression drastically altered the sporulation frequency in R20291 and 630Δerm, we had anticipated a stronger sporulation phenotype in the corresponding dccA mutants. However, there are many additional DGCs encoded in the C. difficile genome as well as many PDEs, and these likely contribute to the intracellular concentration of c-di-GMP also, as most are catalytically active (10). Given the sheer number of encoded DGCs and that their functions may inherently exhibit redundancy, it is, in retrospect, unsurprising that the deletion of a single GGDEF domain protein does not significantly impact sporulation. Any contribution of DccA to the intracellular c-di-GMP pool may be masked by the redundant functions of similar proteins. As such, we previously found that the overexpression of DccA resulted in modest increases in the transcript levels of several encoded PDEs, suggesting that C. difficile is able to somewhat compensate for high levels of intracellular c-di-GMP (55). It is also possible that changes in c-di-GMP levels upon the loss of DccA are compensated for by the up-regulation or activation of other DGCs, the downregulation or inhibition of PDEs, or
both. Importantly, previous studies have shown that distinct DGCs control different c-di-GMP-regulated phenotypes, suggesting that localized pools of c-di-GMP influence only a subset of phenotypes (56, 57). It is plausible that the deletion of one or more of the other encoded DGCs in C. difficile could result in a stronger impact on the sporulation frequency. A recent report describes that the overexpression of a phosphodiesterase containing an EAL domain increases sporulation and that the deletion of that PDE decreases sporulation in C. difficile UK1, an epidemic strain that is nearly identical to R20291, corroborating our findings (58).

Interestingly, two early C. difficile sporulation regulators that are orthologous to the B. subtilis SinR transcriptional repressor regulate C. difficile intracellular c-di-GMP levels. Null mutations in the two C. difficile SinR orthologs, known as SinRR’ in R20291 and CD2214-CD2215 in 630Δerm, result in increased dccA transcription and c-di-GMP levels and an asporogenous phenotype (20, 59). Furthermore, the deletion of CD2214-CD2215 resulted in the differential expression of additional DGC and PDE genes in C. difficile 630Δerm (20). It will be intriguing to determine if the effect of SinRR’ on C. difficile sporulation occurs through alteration of c-di-GMP levels by regulating DGC/PDE gene expression or if SinRR’ affects sporulation initiation through multiple regulatory pathways.

Identification of the c-di-GMP effector(s) that mediates c-di-GMP-dependent sporulation regulation in C. difficile is of great interest. A variety of c-di-GMP receptors that directly bind to c-di-GMP have been characterized in bacteria. These encompass a number of protein-based receptors, including proteins containing degenerate GGDEF and/or EAL domains, and two distinct types of riboswitches that alter downstream gene expression in response to c-di-GMP binding (8). C. difficile encodes a single PilZ domain protein (6), a domain that often directly binds c-di-GMP (60, 61), and a type IV pilus PilIB ATPase similar to orthologs that have been shown to bind c-di-GMP (62), but there are no other known or predicted c-di-GMP protein receptors reported in C. difficile (8). C. difficile encodes at least 11 functional riboswitches that alter gene expression in response to c-di-GMP and contains 5 additional predicted riboswitches (15, 55). None of these riboswitches appear to affect the expression of sporulation-related genes; however, the conditions used in this study do not support efficient sporulation initiation in C. difficile (55). Performing transcriptomics under conditions that favor sporulation may provide insights into which regulatory pathway(s) or factor(s) is required to mediate this c-di-GMP-dependent response.

The regulatory pathway that c-di-GMP utilizes to influence sporulation remains unknown. The decrease in spore formation mediated by dccA overexpression does not appear to signal through CodY, CcpA, the Opp or App oligopeptide permeases, RstA, or the CD1492 and CD2492 phosphotransfer proteins. Although the effect of DccA-mediated inhibition of sporulation was slightly decreased in the sigD mutant, c-di-GMP is unlikely to signal solely through SigD under these conditions. Given that we know that c-di-GMP directly affects sigD transcription in C. difficile through the Cdi-1-3 riboswitch (13, 15, 50), it is attractive to hypothesize that SigD might have a role in sporulation. It may be possible that decreased levels of SigD are necessary for c-di-GMP to affect sporulation; in this case, testing the c-di-GMP-dependent effects on sporulation in a sigD mutant or in JIR8094, a phase-off, nonmotile strain with low SigD levels, may not directly answer this question. Thus far, there is no published evidence of a regulatory role for SigD in sporulation (39, 49, 55).

The impact of c-di-GMP on sporulation has been investigated in only a few other spore-forming bacteria. Interestingly, using an mCherry reporter fused to a c-di-GMP-regulated riboswitch, high c-di-GMP levels were observed in sporulating B. subtilis cells, suggesting a correlation (63), but the impact of c-di-GMP on B. subtilis sporulation has remained relatively unexplored. Studies in Bacillus thuringiensis and Bacillus anthracis found no effect on the sporulation efficiency when any of the catalytically active GGDEF/EAL/HD-GYP-encoding genes were individually deleted (64, 65). These studies in Bacillus sp. further underscore the differences between C. difficile and other
endospore-forming bacteria in their early sporulation regulatory networks. Finally, direct c-di-GMP regulation of sporation has been identified only in *Streptomyces* sp., where c-di-GMP inhibits spore formation directly by antagonizing the sporation-specific sigma factor WhiG and binding directly to the transcriptional regulator BldD (66, 67). The contribution of c-di-GMP to sporation remains an understudied field.

Utilizing c-di-GMP to inhibit sporation may be advantageous to *C. difficile*, as c-di-GMP can be rapidly degraded when environmental and intracellular conditions favor sporation. The c-di-GMP metabolic activity of a protein is often controlled by the corresponding domains encoded within the same protein. Identifying the DGCs and PDEs that affect *C. difficile* sporation and investigating the function of their associated domains may reveal the environmental and intracellular signals that promote or delay sporation. Finally, the finding that c-di-GMP is a regulator of early sporation events in *C. difficile* creates new research opportunities for discovering the potentially novel regulatory pathways, c-di-GMP effectors, and molecular mechanisms that control spore formation in this significant pathogen.

**MATERIALS AND METHODS**

**Bacterial strains and growth conditions.** The bacterial strains and plasmids used for this study are listed in Table 2. *Clostridioioides difficile* was routinely cultured in brain heart infusion-supplemented (BHIS) medium in a 37°C vinyl anaerobic chamber (Coy) with an atmosphere of 5% CO₂, 10% H₂, and 85% N₂ (68). *C. difficile* cultures were supplemented with 2 to 10 μg/ml thiamphenicol as necessary for plasmid maintenance. *Escherichia coli* strains were grown aerobically at 37°C in LB with 100 μg/ml ampicillin and/or 10 to 20 μg/ml chloramphenicol, and counterselection against *E. coli* after conjugation with *C. difficile* was performed using 100 μg/ml kanamycin (13).

**Strain and plasmid construction.** *C. difficile* strains 630 (GenBank accession no. NC_009089.1) and R20291 (GenBank accession no. FNS45816.1) were used as the basis for primer design and PCR amplification (oligonucleotides used in this study are listed in Table 3). The dccA mutants were constructed using the pseudo-suicide vectors pMSR and pMSR0 (42). Upstream and downstream homology regions were amplified from the 630Δerm genome with primer pairs R2928/R2929 and R2930/R2931, respectively. The fragments were Gibson assembled (New England BioLabs (NEB)) into SalI/Xhol-digested pMSR to create pMSR::ΔdccA. Similar fragments were amplified from the R20291 chromosome using the same primers and assembled into pMSR0 to create pMSR0::ΔdccA. Chloramphenicol-resistant colonies were confirmed by PCR with plasmid-specific primer pair R838/R1832 (pMSR) or R2743/R2744 (pMSR0).

To create the dccA mutants, the pMSR::ΔdccA and pMSR0::ΔdccA plasmids were transformed into *E. coli* HB101 pRK24 for conjugation with *C. difficile* 630Δerm and R20291, respectively. Subsequent steps were done essentially as previously described (42). Briefly, large thiamphenicol- and kanamycin-resistant colonies, which presumably had integrated the plasmid into the chromosome, were streaked again on BHIS medium with 100 ng/ml anhydrotetracycline (ATc) to induce the expression of the toxin gene and eliminate bacteria that still contained the vector. Colonies were screened for the 0.8-kb deletion of dccA using primer pair R2926/R2927 (Fig. 7A). Genomic DNA was isolated from potential mutants, and the dccA region was PCR amplified using primer pair R2926/R2927 and sequenced to confirm the integrity of the sequence.

The 630Δerm and R20291 dccA mutants were complemented using a *Bacillus subtilis* donor strain, BS49, carrying the conjugal transposon Tn916 to transfer the dccA gene driven by its native promoter, which is encoded upstream of CD1421 (MC1959). To create the plasmid carrying the Tn916::P_{cdccA} dccA construct (pMC1094), P_{cdccA} and dccA were spliced by overlapping PCR using primer pairs oMC2910/2911 and oMC2912/2913 and Gibson assembled into BamHI/SphI-digested pSMB47. Erythromycin-resistant colonies were confirmed by PCR with primer pair CD1420qf/CD1420qr (Fig. 7B).

To create the 630Δerm ΔCD2492 CD2492:erm double mutant, the Targetron-based group II intron from pCE240 was retargeted using the targeting site reported previously by Underwood et al. to create pmC336 (69). Briefly, the CD2492-targeted intron was amplified using primers oMC317, oMC318, oMC319, and EBSu and TA cloned into pCR2.1 to create pMC330. A BsrGI/HindIII-digested fragment containing the CD2492-targeted intron was subcloned into pCE240 to create pMC333. Finally, an SphI/SfoI-digested fragment containing the CD2492-targeted intron was subcloned into pMC123 to create pmC336. The resulting construct, pmC336, was conjugated into the 630Δerm CD1492 strain (MC674), and erythromycin-resistant mutants were screened for the 2-kb Targetron insertion within CD2492 using primer pair oMC309/338 (Fig. 7C). Notably, the targeting site was not located in the 254a site within the CD2492 coding region noted by Underwood et al. (69) but rather was located at 318s (data not shown).

**Sporation assays.** *C. difficile* strains were grown overnight in BHIS medium supplemented with 0.1% taurocholate and 0.2% fructose to aid in spore germination and prevent spore formation, respectively (70, 71). Thiamphenicol (5 μg/ml) was included for plasmid maintenance when necessary. When strains reached mid-exponential phase (optical density at 600 nm [OD₆₀₀] of ~0.5), 250-μl aliquots were applied to the surface of 70/30 agar containing 2 μg/ml thiamphenicol and 0 to 0.5 μg/ml nisin (71). After 24 h of growth, ethanol-resistant sporation assays were performed as previously described (48, 72). Briefly, cells were scraped from the plate surface and suspended in BHIS medium to an OD₆₀₀ of ~1. To enumerate
TABLE 2  Bacterial strains and plasmids

| Strain (lab annotation) or plasmid | Relevant genotype or feature(s) | Source and/or reference(s) |
|-----------------------------------|--------------------------------|---------------------------|
| **Strains**                       |                                |                           |
| *E. coli*                         |                                |                           |
| HB101                             | \( F^- \), mcrB, hsdS20(r<sub>R</sub> K<sub>mB</sub>), recA13, leuB6, ara-14, proA2, lacY1, galK2, xyl-5, mtl-1, rpsL20 | B. Dupuy                  |
|                                   | pRK24                          |                           |
| B. *subtilis*                     |                                |                           |
| BS49                              | CU2189::Tn916                   | P. Mullany                |
| MC1959                            | BS49 Tn916::P<sub>CD1421-dccA</sub> | This study               |
| C. *difficile*                    |                                |                           |
| 630Δerm                           | Erm<sup>+</sup> derivative of strain 630 | N. Minton; 36 |
| R20291                            |                                | 35                        |
| JIR8094                           | Erm<sup>+</sup> derivative of strain 630 (630E) | B. Dupuy; 75 |
| JIR8094 ccpA::erm                 |                                | 54                        |
| RT526                             | R20291 pMC211                   | 13                        |
| RT527                             | R20291 pDccA                    | 13                        |
| RT539                             | R20291 pDccA<sup>+</sup>        | This study                |
| RT762                             | 630Δerm pMC211                  | This study                |
| RT763                             | 630Δerm pDccA                   | This study                |
| RT764                             | 630Δerm pDccA<sup>+</sup>       | This study                |
| RT993                             | 630Δerm::P<sub>oppB::erm</sub>  | 41                        |
| RT1050                            | 630Δerm pP<sub>oppB::ERM</sub>-UTR-gusA | 41            |
| RT1051                            | 630Δerm pP<sub>oppB::erm</sub>-UTR<sup>70G</sup>-gusA | 41           |
| RT1054                            | 630Δerm::P<sub>oppB::erm</sub>  | 41                        |
| RT1055                            | 630Δerm::P<sub>oppB::erm</sub>  | 41                        |
| RT1075                            | 630Δerm sigD::erm               | 39                        |
| RT2257                            | R20291 ΔcmtT                    | 37                        |
| RT2283                            | R20291 ΔcmtT pMC211             | This study                |
| RT2284                            | R20291 ΔcmtT pDccA              | This study                |
| RT2656                            | R20291 ΔcddA                    | This study                |
| RT2703                            | 630Δerm ΔcddA                   | This study                |
| MC307                             | 630Δerm oppB::erm oppA::erm     | 33                        |
| MC364                             | 630Δerm codY::erm               | 31                        |
| MC391                             | 630Δerm rstA::erm               | 39                        |
| MC802                             | 630Δerm ΔCD1492 CD2492::erm     | This study                |
| MC864                             | 630Δerm sigD::erm pMC211        | This study                |
| MC865                             | 630Δerm sigD::erm pDccA         | This study                |
| MC924                             | 630Δerm oppB::erm oppA::erm pMC211 | This study        |
| MC925                             | 630Δerm oppB::erm oppA::erm pDccA | This study       |
| MC926                             | 630Δerm rstA::erm pMC211        | This study                |
| MC927                             | 630Δerm rstA::erm pDccA         | This study                |
| MC928                             | 630Δerm ΔCD1492 CD2492::erm pMC211 | This study        |
| MC929                             | 630Δerm ΔCD1492 CD2492::erm pDccA | This study        |
| MC943                             | JIR8094 pMC211                  | This study                |
| MC944                             | JIR8094 pDccA                   | This study                |
| MC945                             | JIR8094 ccpA::erm pMC211        | This study                |
| MC946                             | JIR8094 ccpA::erm pDccA         | This study                |
| MC947                             | 630Δerm codY::erm pMC211        | This study                |
| MC948                             | 630Δerm codY::erm pDccA         | This study                |
| MC1960                            | 630Δerm ΔcddA Tn916::P<sub>CD1421-dccA</sub> | This study        |
| MC1961                            | R20291 ΔcddA Tn916::P<sub>CD1421-dccA</sub> | This study        |
| **Plasmids**                      |                                |                           |
| pRK24                             | Tra<sup>+</sup>, Mob<sup>+</sup>, bla, tet | 76                        |
| pSMB47                            | Tn916 integrational vector, catP, 77 |
| pCR2.1                            | bla, kan                       | Invitrogen                |
| pMSR                              | Allelic exchange in *C. difficile* 630 | 42                        |
| pMSR0                             | Allelic exchange in *C. difficile* 20291 | This study                |
| pMSR::ΔcddA                       | dccA deletion construct in pMSR | This study                |
| pMSR0::ΔcddA                      | dccA deletion construct in pMSR0 | This study                |
| pCE240                            | *C. difficile* Targetron construct based on pJIR750ai (group II intron, ermB::RAM ltrA), catP | C. Ellermeier     |
| pMC330                            | pCR2.1 with group II intron targeted to CD2492 | This study                |
| pMC333                            | pCE240 with CD2492-targeted intron | This study                |

(Continued on next page)
vegetative cells, the cell suspensions were serially diluted in BHIS medium and plated onto BHIS plates. Simultaneously, 0.5-ml aliquots of the cell suspensions were mixed thoroughly with 0.3 ml 95% ethanol (final concentration, 28.5% ethanol) and incubated for 15 min to eliminate all vegetative cells. Ethanol-treated cells were then serially diluted in 1/10 C2-phosphate-buffered saline (PBS) with 0.1% taurocholate and plated onto BHIS medium supplemented with 0.1% taurocholate. Total CFU were enumerated after at least 24 h of growth, and the sporulation frequency was calculated as the number of ethanol-resistant spores divided by the total number of vegetative and ethanol-resistant spores combined. A spo0A mutant (MC310) was used as a negative control in all assays.

Phase-contrast microscopy. Phase-contrast microscopy was performed at H+4 as described previously (33). Briefly, cells were concentrated by pelleting 0.5 ml of the cell suspension, and the concentrated cell suspension was applied to a 0.7% agarose pad on a slide. Cells were imaged with a 100× Ph3.

| Table 2 (Continued) |
|----------------------|
| Strain (lab annotation) or plasmid | Relevant genotype or feature(s) | Source and/or reference(s) |
| pMC336 | pMC123 with CD2492-targeted intron | This study |
| pMC211 | E. coli-C. difficile shuttle vector; bla catP | 13, 33 |
| pDccA | CD1420 from 630 in pMC211 | 13 |
| pDccAmut | CD1420 (AADEF) in pMC211 | 13 |
| pMC1094 | P<sub>CD1421</sub>-dccA in pSMB47 | This study |
| p<sub> pilA1</sub>-UTR-gusA | | 41 |
| p<sub> pilA1</sub>-UTR<sup>del</sup>-gusA | | 41 |

| Table 3 Oligonucleotides |
|--------------------------|
| Primer | Sequence (5′→3′) | Reference, source, or use (reference) |
| CD1420qF | 5′-AAGAAGACTCCCTGATAATATTGCTAA | 13 |
| CD1420qR | 5′-ACATTCCAATAGCTTGTAGTATCTTT | 13 |
| EBSu | 5′-CGAAATTAGAAACCTGGTCTAGAAC | Sigma-Aldrich |
| oMC44 | 5′-CTAGCCTGTCACTGTCTCACATC | Forward primer for rpoC (34) |
| oMC45 | 5′-CCAGTCCTCCTGGTACAACTA | Reverse primer for rpoC (34) |
| oMC309 | 5′-GGAGAATAACAGAGTTGTTGATTTCCC | Forward primer for CD2492:erm verification |
| oMC317 | 5′-AAAAGCTTTTGGACACCGTCGATGGAATGCTTTAATGCTGCGCCCACTAGCGG | IBS for CD2492-targeted intron |
| oMC318 | 5′-CAGATGTGAAATGTTGCTGATAACAGATAATCTCTAATCTCAATCTTACC | EBS1 for CD2492-targeted intron |
| oMC319 | 5′-CGAAGATTCTTATTTCGATATATCACTGTAGAAGGAGGTCT | EBS2 for CD2492-targeted intron |
| oMC338 | 5′-TCCATTGCTCCTATTGGAAACTGA | Reverse primer for CD2492:erm verification (39) |
| oMC339 | 5′-GGGAAATAATTTTCTCCTCCCAT | Forward primer for sigE (CD2643) (33) |
| oMC340 | 5′-TGACCTTACCTCTGGTGTTTCTAGCG | Reverse primer for sigE (CD2643) (33) |
| oMC2910 | 5′-GACACACCCGTCGTTGATCCCCCATCTCATGAAATATTTCATTTCTCTTATTCTTCTT | Forward primer for PCD1421 |
| oMC2911 | 5′-CTTAAAAACATATTGATTTCTCCTAAAAATAACATGGTTGATTTCC | Reverse primer for PCD1421 |
| oMC2912 | 5′-CAAATTTAAGGTATTTCTTTGCTATCCAATTC ATTAAAACTATTTTTTTT | Forward primer for dccA (CD1420) |
| oMC2913 | 5′-CCGGCGCAAGAATGGTGCTACGTAATCTCATTTTCTTATCAAA | Reverse primer for dccA (CD1420) |
| R838 | 5′-GTAAACGAGCCGCCAGT | Reverse screening primer for pMSR |
| R1832 | 5′-TATTCCTGATGTCCTGACT | Forward screening primer for pMSR |
| R2743 | 5′-GTGTTATCAATGTCGACTACGAT | Forward screening primer for pMSR0 |
| R2744 | 5′-GTGGAACCTATGCAAGGTTTAC | Reverse screening primer for PMSR0 |
| R2926 | 5′-CTGATATAAGAAAATCTTCTTAATAGAAG | Forward screening primer for dccA chromosomal deletion |
| R2927 | 5′-TCCATGAACCTCATCATATGTGTATCC | Reverse screening primer for dccA chromosomal deletion |
| R2928 | 5′-TCGCCATCTCTAGATGTGAAGATGAATGTAATGAAATGAACATGAG | Forward primer for upstream fragment of dccA deletion construct |
| R2929 | 5′-CAAACTCTCATTTGTTCTTCTCATATTA | Reverse primer for upstream fragment of dccA deletion construct |
| R2930 | 5′-GTATTTTATTTTTGAGAAATTTATGAGGAAAACAAGAAAATAATTGGATAAAATG | Forward primer for downstream fragment of dccA deletion construct |
| R2931 | 5′-ATGGTCCGGCGCTCGAGAGATGTTATCATCATCATTGCTACC | Reverse primer for downstream fragment of dccA deletion construct |
on 70:30 sporulation agar at H12 and DNase I treated as previously described (13, 29, 33, 34). cDNA was synthesized using random hexamers, and quantitative real-time-PCRs were performed in triplicate (41, 74). Briefly, strains were grown on 70:30 sporulation agar supplemented with 2 μg/ml thiamphenicol and 0.5 μg/ml nisin and harvested at H12. Total protein from the cell lysates was quantitated using the Pierce Micro bicinchoninic acid (BCA) protein assay kit (Thermo Scientific). 2.5 μg of total protein was separated by electrophoresis on a precast 4 to 20% TGX stain-free gradient gel (Bio-Rad), and total protein was imaged using a ChemiDoc system (Bio-Rad). Protein was transferred to a nitrocellulose membrane, and Western blot analysis was performed with mouse anti-Spo0A antibody and goat anti-mouse conjugated with Alexa Fluor 488 (Invitrogen) as the secondary antibody. Imaging and densitometry were performed with a ChemiDoc system and Image Lab software (Bio-Rad), respectively, for three independent experiments.

Quantitative reverse transcription-PCR analysis. RNA was isolated from C. difficile strains grown on 70:30 sporulation agar supplemented with 2 μg/ml thiamphenicol and 0.5 μg/ml nisin and harvested at H12. Total protein from the cell lysates was quantitated using the Pierce Micro bicinchoninic acid (BCA) protein assay kit (Thermo Scientific). 2.5 μg of total protein was separated by electrophoresis on a precast 4 to 20% TGX stain-free gradient gel (Bio-Rad), and total protein was imaged using a ChemiDoc system (Bio-Rad). Protein was transferred to a nitrocellulose membrane, and Western blot analysis was performed with mouse anti-Spo0A antibody and goat anti-mouse conjugated with Alexa Fluor 488 (Invitrogen) as the secondary antibody. Imaging and densitometry were performed with a ChemiDoc system and Image Lab software (Bio-Rad), respectively, for three independent experiments.

Western blotting. The indicated strains were grown on 70:30 sporulation agar supplemented with 2 μg/ml thiamphenicol and 0.5 μg/ml nisin and harvested at H12. Total protein from the cell lysates was quantitated using the Pierce Micro bicinchoninic acid (BCA) protein assay kit (Thermo Scientific). 2.5 μg of total protein was separated by electrophoresis on a precast 4 to 20% TGX stain-free gradient gel (Bio-Rad), and total protein was imaged using a ChemiDoc system (Bio-Rad). Protein was transferred to a nitrocellulose membrane, and Western blot analysis was performed with mouse anti-Spo0A antibody and goat anti-mouse conjugated with Alexa Fluor 488 (Invitrogen) as the secondary antibody. Imaging and densitometry were performed with a ChemiDoc system and Image Lab software (Bio-Rad), respectively, for three independent experiments.

β-Glucuronidase reporter assays. β-Glucuronidase assays were performed as previously detailed (41, 74). Briefly, strains were grown on 70:30 sporulation agar as indicated above and harvested at H12 and H15 by scraping the plates and suspending the cells in BHIS medium to an OD600 of ~0.5 to 0.7. Two 1-ml aliquots were pelleted and stored at −20°C overnight. The pellets were suspended in 0.8 ml Z buffer and 0.05 ml 0.1% SDS. The samples were vortexed, incubated at 37°C for 5 min, and then chilled on ice for 5 min. After a 1-min incubation at 37°C to warm the samples up to room temperature, the enzymatic reaction was started with the addition of 100 μl of 40 μg/ml β-nitrophenol–β-D-gluconoride and stopped with 0.4 ml 1 M Na2CO3. Cell debris was pelleted, and the A420 and A550 were measured using a BioTek Synergy H1 plate reader. Specific activity was normalized by the OD600. Three independent biological replicates were used to calculate the means and standard errors of the means from at least three independent experiments.

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