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The Second Messenger Cyclic Di-GMP Regulates *Clostridium difficile* Toxin Production by Controlling Expression of *sigD*

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The Gram-positive obligate anaerobe *Clostridium difficile* causes potentially fatal intestinal diseases. How this organism regulates virulence gene expression is poorly understood. In many bacterial species, the second messenger cyclic di-GMP (c-di-GMP) negatively regulates flagellar motility and, in some cases, virulence. c-di-GMP was previously shown to repress motility of *C. difficile*. Recent evidence indicates that flagellar gene expression is tightly linked with expression of the genes encoding the two *C. difficile* toxins TcdA and TcdB, which are key virulence factors for this pathogen. Here, the effect of c-di-GMP on expression of the toxin genes *tcdA* and *tcdB* was determined, and the mechanism connecting flagellar and toxin gene expressions was examined. In *C. difficile*, increasing c-di-GMP levels reduced the expression levels of *tcdA* and *tcdB*, as well as that of *tcdR*, which encodes an alternative sigma factor that activates *tcdA* and *tcdB* expression. We hypothesized that the *C. difficile* orthologue of the flagellar alternative sigma factor SigD (FliA; σ^{28}) mediates regulation of toxin gene expression in response to c-di-GMP. Indeed, ectopic expression of *sigD* in *C. difficile* resulted in increased expression levels of *tcdR*, *tcdA*, and *tcdB*. Furthermore, *sigD* expression enhanced toxin production and increased the cytopathic effect of *C. difficile* on cultured fibroblasts. Finally, evidence is provided that SigD directly activates *tcdR* expression and that SigD cannot activate *tcdA* or *tcdB* expression independent of TcdR. Taken together, these data suggest that SigD positively regulates toxin genes in *C. difficile* and that c-di-GMP can inhibit both motility and toxin production via SigD, making this signaling molecule a key virulence gene regulator in *C. difficile*.

Clostridium difficile, a Gram-positive obligate anaerobe, is a leading cause of nosocomial infections in North America, Europe, and Australia (1–5). *C. difficile* infections (CDIs) are extremely costly to treat and represent a substantial economic burden (6–8). The bacterium is transmitted between hosts as a dormant spore, a metabolically inactive form that is resistant to oxygen and to many common disinfectants. CDI often occurs following antibiotic use, which disrupts the intestinal microbiota and allows *C. difficile* spores to germinate in and colonize the intestine. In the large intestine, *C. difficile* produces cytotoxins that cause a range of disease manifestations, including diarrhea and potentially lethal pseudomembranous colitis.

Two Rho GTPase-glucosylating cytotoxins, TcdA and TcdB, are key virulence factors of *C. difficile*, and both have been shown to play important roles in disease by using animal models (9, 10). Several regulators of expression of the *tcdA* and *tcdB* genes have been identified to date. Two regulators, TcdR and TcdC, are encoded in the pathogenicity locus that also encodes TcdA and TcdB. TcdR is an alternative sigma factor that positively regulates transcription of *tcdA* and *tcdB* (11, 12). TcdC may function as an anti-sigma factor that impedes the activity of TcdR, although some reports have found that TcdC does not affect toxin production (13–17). In addition, the global transcriptional regulator CodY represses *tcdA*, *tcdB*, and *tcdR* expression during exponential growth in response to GTP and branched-chain amino acids and acts primarily by controlling expression of *tcdR* (18, 19). The catabolite control protein CcpA represses toxin gene expression (but not *tcdR* expression) in response to glucose (20, 21). SigH, an alternative sigma factor involved in controlling gene expression during entry into stationary phase, also affects toxin gene expression (22), although the regulatory mechanisms involved have not been determined. Finally, Spo0A, a regulator of sporulation initiation, has been implicated in the regulation of toxin gene expression, but these findings remain controversial (23–25).

Flagella have been shown to play a significant role in the colonization of host tissues by a wide variety of bacteria (26–30). Flagella and motility have been implicated in the ability of *C. difficile* to cause disease in animal models, but there are conflicting data on the exact way in which flagella impact virulence. One study suggested a positive influence of flagella on intestinal colonization using animal models but relied on comparisons of nonisogenic isolates (31). Studies of isogenic *C. difficile* mutants with insertions in the flagellar filament genes *fliC* and *fliD*, which encode flagellin and flagellar cap proteins, respectively, suggested that flagella and/or motility is detrimental for attachment to host cells *in vitro* and for virulence in hamsters (32, 33). However, the *fliC* and *fliD* mutants produced more TcdA and TcdB toxins (33), so the specific contribution of FliC and FliD to virulence is unclear. A recent study showed that mutations in other flagellar genes (*fliF*, *fliG*, *fliM*, *flhB*, and *sigD*), each of which caused a loss of motility, resulted in significantly decreased toxin gene expression; a subset of these nonmotile mutants was tested in the hamster model of CDI, and some but not all of the mutants were attenuated for virulence (34). Although the exact regulatory relationship between flagellar and toxin gene expressions is unknown, it is clear that mutation of flagellar genes impacts the ability of *C. difficile* to cause disease, whether by a direct effect on flagellum-mediated

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colonization, an indirect effect on toxin production, or a combination of the two.

Recently, we reported that the bacterial second messenger cyclic diguanylate (c-di-GMP) inhibits swimming motility of *C. difficile* (35). The c-di-GMP signaling molecule negatively regulates the flagellar motility of a large number of bacteria by diverse mechanisms, including transcriptional, posttranscriptional, and posttranslational control (36–44). In *C. difficile*, regulation of motility by c-di-GMP likely occurs through a direct effect of c-di-GMP on the expression of a putative large flagellar operon, the *flgB* operon, via a c-di-GMP riboswitch (45). This riboswitch, named Cd1, was shown previously to respond to c-di-GMP levels in a heterologous host (*Bacillus subtilis*), and *in vitro* analysis of Cd1 RNA indicates that Cd1 interacts directly with c-di-GMP to trigger premature transcription termination (45). A recent study that focused on identifying regulatory RNAs in *C. difficile* experimentally confirmed the expression of Cd1 riboswitch RNA corresponding to the ~160-nucleotide terminated transcript and demonstrated that elevated c-di-GMP levels increase the abundance of the terminated Cd1 riboswitch RNA *in vivo* (46). These findings support a direct effect of c-di-GMP on the transcription of the *flgB* operon, providing a mechanism by which c-di-GMP represses motility of *C. difficile*.

The putative *flgB* operon contains genes predicted to encode proteins involved in the early steps of flagellar biosynthesis, such as flagellar hook, basal body, MS ring, motor, and other assembly proteins. Among the genes in the *flgB* operon is *sigD*, which is predicted to encode an alternative sigma factor. The orthologues of SigD present in many Gram-negative bacteria (in which orthologues are typically named FliA or σ^{28}) and Gram-positive bacteria positively regulate the expression of flagellar genes in a hierarchical manner that allows the bacteria to link flagellar gene expression to the assembly of the flagellum (47–55). Best characterized in Gram-negative bacteria, and in *Salmonella enterica* serovar Typhimurium in particular, hierarchical control of flagellar gene expression by FliA is accomplished through control of *fliA* transcription and through posttranslational control of FliA by the anti-sigma factor FlgM (56–62). Specifically, FliA activity is antagonized by FlgM until the hook-basal body structure is assembled, and FlgM is secreted from the cell through the assembled complex (48, 63–65). The FlgM concentration in the cell decreases, allowing FliA, in conjunction with RNA polymerase, to activate its target promoters, typically those controlling the expression of "late-stage" flagellar components such as flagellin, flagellar motor proteins, and chemotaxis proteins. In the model Gram-positive bacterium *B. subtilis*, SigD similarly positively regulates genes involved in flagellum synthesis, motility, and chemotaxis in a hierarchical fashion involving antagonism of SigD activity by FlgM (66–72). The genetic organization of the flagellar locus of *C. difficile* differs from those of both Gram-negative bacteria and *B. subtilis*, and much remains to be experimentally demonstrated regarding the operon arrangements, regulatory relationships between flagellar genes, and functionality of orthologues of known flagellar gene regulators.

Previously, we showed that *C. difficile* with elevated intracellular levels of c-di-GMP has reduced expression levels of *flgB* and *sigD* (35). Elevated c-di-GMP levels also resulted in reduced expression levels of *flgM*, which lies upstream of the *flgB* operon and is thus expected to be indirectly impacted by c-di-GMP. We predicted that SigD mediates the effect of high c-di-GMP levels on the expression of late-stage flagellar genes. Because c-di-GMP regulates the expression of flagellar genes, and flagellar gene mutations impact the expression of toxin genes, we hypothesize that c-di-GMP also regulates the expression of the toxin genes. Indeed, *C. difficile* with a mutation in *sigD* (annotated *fliA* and named accordingly by Aubry et al.) was previously shown to be nonmotile and to express lower levels of *tcdR*, *tcdA*, and *tcdB* (34). In this study, we investigated the effect of altering intracellular c-di-GMP levels on toxin production by *C. difficile* and assessed the role of SigD in regulating toxin gene expression in response to c-di-GMP. Our results provide evidence that c-di-GMP is a key regulator of virulence genes in *C. difficile* and that the alternative sigma factor SigD plays a central role in this regulation.

MATERIALS AND METHODS

Bacterial strains and growth conditions. Table S1 in the supplemental material lists the bacterial strains and plasmids used in this study. *C. difficile* strains were grown at 37°C under anaerobic conditions in BHIS or TY medium as specified previously (35, 73). *Escherichia coli* and *Bacillus subtilis* were grown in LB broth at 37°C with aeration (74). When appropriate, the following antibiotics were used at the indicated concentrations: ampicillin (Amp) at 100 μg/ml, chloramphenicol (Cm) at 10 μg/ml, thiamphenicol (Tm) at 10 μg/ml, kanamycin (Km) at 100 μg/ml, and erythromycin (Erm) at 5 μg/ml. Nisin was used for induction of gene expression at a final concentration of 1 μg/ml unless otherwise indicated.

DNA manipulations and strain construction. Table S2 in the supplemental material lists the oligonucleotides used in this study. *C. difficile* 630 genomic DNA was used as the template for all PCRs unless otherwise specified. All DNA modification enzymes were purchased from New England BioLabs.

For expression of *sigD* in *C. difficile*, *sigD* (CD0266) was amplified by PCR using primers sigDCdeF and sigDCdeR, which introduced BamHI and PstI restriction sites, respectively. The digested fragment was cloned into similarly digested pMC-Pcpr (35). Clones were confirmed by PCR with primers pUCmcsF and m13r, resulting in plasmid pSigD. Plasmid pSigD was introduced into *E. coli* HB101(pRK24) cells by electroporation. The HB101(pRK24) donor strain was then mated with *C. difficile* strains 630 and JIR8094, resulting in the transfer of pSigD into *C. difficile*. Transconjugants were selected on BHIS medium with 10 μg/ml thiamphenicol (BHIS-Tm) supplemented with kanamycin to prevent growth of the *E. coli* donor strain. Isolates were confirmed by PCR with primers pUCmcsF and m13r (to confirm the presence of the plasmid) and with primers tcdBqF and tcdBqR (to confirm *C. difficile*), resulting in strains RT731 and RT747.

To generate a *C. difficile tcdR* mutant, the Targetron method was used (75, 76) but with a modified set of template and delivery plasmids (77). The Targetron was targeted to a sequence within *tcdR*, AAAAAAGCG ATG. The targeted intron fragment was generated by PCR, amplifying one portion from pBL64 using primers EBSuniv and tcdRibs1 and the other portion from pBL65 using primers tcdRebs1 and tcdRebs2. The PCR products were combined and used as the template in a second round of PCR with primers tcdRibs1 and tcdRebs1, which allowed splicing of the two initial PCR products into a single targeted intron fragment. The intron fragment was digested with BsrGI and HindIII and cloned into pBL100. The resulting plasmid, pBL100::*tcdR*::*ermB*, was confirmed by PCR and sequencing and then introduced into *E. coli* HB101(pRK24). Plasmid pBL100::*tcdR*::*ermB* was transferred into erythromycin-sensitive *C. difficile* 630ΔErm by conjugation. Transconjugants were selected on BHIS-Tm-Km medium. Isolates with Targetron insertions were selected by screening transconjugants for resistance to erythromycin and then confirmed to have an insertion in *tcdR* by PCR with primers tcdRqF and tcdRrev. These primers yielded a 323-bp product from *C. difficile* with a wild-type *tcdR* allele and a ~2.3-kb product from the *C. difficile* 630ΔErm *tcdR*::*ermB* mutant (see Fig. S5A in the supplemental material). The *C.*

difficile tcdR::*ermB* mutant was further confirmed by Western blotting for TcdA, the production of which is positively regulated by TcdR (see Fig. S5B in the supplemental material) (11, 12). Plasmids pMC-Pcpr and pSigD were introduced into the *tcdR*::*ermB* mutant by conjugation, as described above.

The *B. subtilis lacZ* reporter strains were made in multiple steps. First, genomic DNA from a *B. subtilis* strain containing a plasmid insertion (pLC5, which confers Cm resistance) in *sigD* (50) was transformed into MC202 (78). Cm-resistant colonies were screened for the incorporation of plasmid pLC5 into *sigD* by PCR, and strain RT838 was obtained. The following promoters of interest were amplified by PCR from *C. difficile* 630 chromosomal DNA (using the primers indicated in parentheses): P_{tcdA} (PtcdAFB and PtcdARH), P_{tcdB} (PtcdBFB and PtcdBRH), P_{tcdR} (PtcdRFB and PtcdRRH), and P_{flgM} (PflgMFB and PflgMRH). The promoter fragments were cloned into pHK23, upstream of the promoterless *lacZ* gene, using BamHI and HindIII sites introduced by using the primers. Correct clones were confirmed by PCR, and the corresponding plasmids were named pRT822 to pRT825. The P_{cpr}::*sigD* fusion was amplified from pSigD by using primers PcprF-XbaI and sigDR-XbaI and then cloned into the XbaI site in plasmids pRT822 to pRT825. PCR was used to identify plasmids with the P_{cpr}::*sigD* insert in the orientation opposite that of the promoter-*lacZ* fusion (i.e., with divergent transcription). The resulting plasmids with both the promoter-*lacZ* and P_{cpr}::*sigD* fusions, pRT828 to pRT831, were transformed into *B. subtilis* strain RT838, leading to recombination of both fusions into the *amyE* locus. The resulting *B. subtilis* reporter strains were confirmed by testing for a loss of spectinomycin resistance and by PCR to detect both fusions.

RNA isolation and quantitative real-time PCR. Measurements of transcript abundance using quantitative reverse transcriptase PCR (qRT-PCR) were used as an indication of expression levels, as described previously (35). RNA was isolated from exponential-phase (optical density [OD] at 600 nm of ~0.6 to 0.8) or early-stationary-phase (OD at 600 nm of ~1.0 to 1.2, approximately 16 h) cultures grown in BHIS medium, as indicated. When strains were grown in TY medium, RNA was isolated from early-stationary-phase cultures (OD at 600 nm of ~2.0 to 2.2). RNA samples were treated with DNase I, cDNA was synthesized, and qRT-PCRs were done as described previously (35). The *rpoC* gene was used as the reference in all experiments (79). Primers were designed by using the PrimerQuest tool from IDT DNA Technologies and follow the naming pattern gene-qF and gene-qR for the forward and reverse primers, respectively (see Table S2 in the supplemental material). Control reaction mixtures lacking reverse transcriptase were included for all templates and all primer sets. At least 3 biological replicates were assayed. The data were analyzed by using the $\Delta\Delta C_T$ method and are expressed as the fold change in the transcript level under the test condition compared to the average for the indicated control and then normalized to the reference gene *rpoC*. Statistical analyses were done by using GraphPad Prism 5 software.

Western blots. *C. difficile* strains with the indicated expression plasmids were grown in BHIS-Tm medium, with or without 1 μg/ml nisin, to exponential phase (OD at 600 nm of ~0.6 to 0.8) or early stationary phase (OD at 600 nm of ~1.0 to 1.2), as indicated for each experiment. Cells were collected by centrifugation from 1 ml of cultures with equivalent optical densities, and the supernatants were filter sterilized. Proteins from cell pellets were suspended directly in Laemmli sample buffer (80). Proteins in the supernatants (from cultures normalized to the OD at 600 nm) were trichloroacetic acid (TCA) precipitated prior to resuspending the proteins in sample buffer. Samples were separated on 4 to 15% SDS-polyacrylamide gels (Bio-Rad). After transfer onto nitrocellulose, the membranes were probed with mouse anti-TcdA or anti-TcdB antibodies (Novus Biologicals) or rabbit anti-CodY antibodies (gift from A. L. Sonenshein) (19). The membranes were then probed with IR800 goat anti-mouse or anti-rabbit secondary antibodies, as appropriate (ThermoFisher). Imaging was done by using an Odyssey Imager (Li-Cor Biologicals). At least 3 biological replicates for each strain were analyzed, and a representative image is shown.

Analysis of cytopathic effects on target cells. *C. difficile* strains with the indicated expression plasmids were grown in BHIS-Tm medium with 1 μg/ml nisin to exponential phase (OD at 600 nm of ~0.6 to 0.8) and early stationary phase (OD at 600 nm of ~1.0 to 1.2). Cells were removed by centrifugation, and the supernatants were filter sterilized. Twofold serial dilutions of the supernatants were applied onto semiconfluent cultures of primary human foreskin fibroblasts grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% newborn calf serum in 96-well plates. After 21 h, the cytopathic effect (CPE) was assessed visually to determine the toxin titer. The toxin titer was defined as the highest dilution for which complete (100%) rounding was observed for the fibroblast cells (9). Four biological replicates were tested, and each biological replicate was assayed in triplicate. The data were analyzed by the Mann-Whitney test. For each biological replicate of the stationary-phase samples, at least one representative well was photographed at a ×40 magnification.

Reporter assays. *B. subtilis* reporter strains were grown overnight at 37°C in LB broth with the appropriate antibiotic selection; diluted 1:50 into the same medium supplemented with 0, 0.5, 1 or 2 μg/ml nisin; and then grown for 6 h at 37°C with aeration. β-Galactosidase activity in *B. subtilis* lysates was measured by using previously described methods and is reported as Miller units (74, 81). Assays were each performed by using 3 biological replicates and were performed independently at least twice. Data were analyzed by using unpaired *t* tests comparing induced to uninduced cultures.

RESULTS

Expression of *tcdA* and *tcdB* is repressed in *C. difficile* with elevated c-di-GMP levels. We previously reported a method for manipulating intracellular c-di-GMP levels in *C. difficile* (35). This method involves the expression of *dccA*, which encodes a diguanylate cyclase (DGC), under the control of the nisin-inducible promoter P_{cpr}. Expression of *dccA* in *C. difficile* was shown to result in 2- to 2,000-fold-increased c-di-GMP levels, depending on the concentration of nisin used to induce *dccA* expression. The intracellular c-di-GMP concentration was unaffected in *C. difficile* expressing $dccA^{mut}$, an allele encoding a catalytically inactive DGC (35). Using this system, c-di-GMP was shown to repress the motility of *C. difficile*, at least in part by decreasing the expression levels of specific flagellar genes (35). Among the genes with reduced transcript levels in *C. difficile* with elevated c-di-GMP levels were *flgB*, *flgM*, and *sigD*, which represent at least two distinct flagellar operons. The flagellar locus consists of a putative 22-kb operon beginning with *flgB* (CD0245) and likely at least two additional operons immediately upstream of the *flgB* operon (see Fig. S1 in the supplemental material). The upstream operon(s) contains "late-stage" flagellar genes, including, among others, the flagellin gene *fliC* and a glycosyltransferase (CD0240) involved in posttranslational modification of flagellin (82). To further examine the extent to which c-di-GMP regulates the expression of genes in the flagellar locus, the *fliC*, *fliI*, *motA*, CD0240, and CD0241 transcripts, which are located throughout the flagellar locus, were chosen to broadly represent the entire locus, and their levels were measured by qRT-PCR. The levels of these transcripts were reduced by over 90% in *C. difficile* with elevated c-di-GMP levels (see Fig. S2 in the supplemental material), indicating that c-di-GMP controls *C. difficile* motility by directly or indirectly repressing the expression of several flagellar genes representing multiple operons.

Because c-di-GMP represses flagellar gene expression (35), and flagellar gene mutations were previously shown to affect the expression of genes in the *C. difficile* pathogenicity locus (33, 34), we

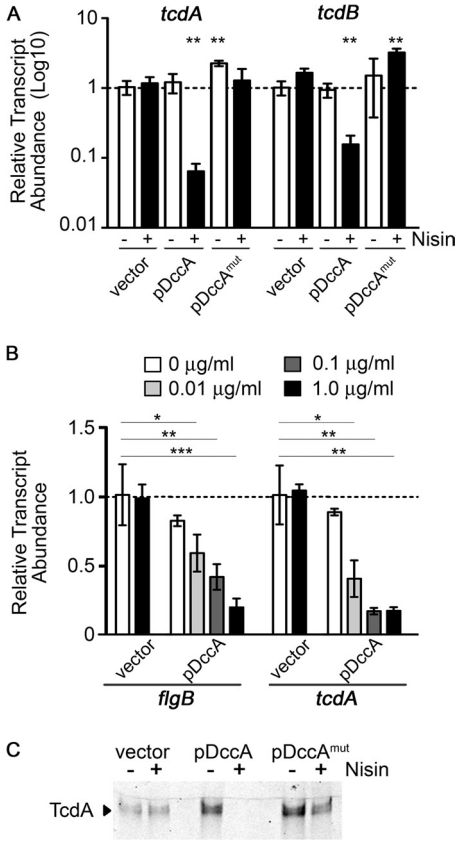

FIG 1 Expression of *tcdA* and *tcdB* in *C. difficile* 630 with elevated c-di-GMP levels. (A) *C. difficile* strains were grown to early stationary phase (OD at 600 nm of ~1.0 to 1.2) in BHIS-Tm medium with or without 1 μg/ml nisin. The levels of the *tcdA* and *tcdB* transcripts in *C. difficile* with the indicated plasmids were measured by qRT-PCR. (B) Transcript abundance of *tcdA* and the *flgB* positive control in *C. difficile* with the vector or pDccA, grown to early stationary phase in BHIS-Tm medium with the indicated concentrations of nisin, was measured by qRT-PCR. In panels A and B, the means and standard deviations are shown, and data were analyzed by one-way analysis of variance and Dunnett's multiple-comparison test comparing values to the average for the induced vector control. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. (C) TCA-precipitated supernatants from cultures of *C. difficile* 630 with the indicated plasmids, grown in BHIS-Tm medium with or without 1 μg/ml nisin to early stationary phase, were probed for TcdA by Western blotting.

predicted that c-di-GMP would also regulate toxin gene expression. We used the nisin-inducible DGC system described above to artificially increase c-di-GMP levels in *C. difficile* and then examined toxin gene expression and toxin production. First, qRT-PCR was used to measure *tcdA* and *tcdB* transcript levels in *C. difficile* 630 with pDccA, pDccA^{mut}, or the vector (pMC-Pcpr) grown in BHIS-Tm medium with or without 1 μg/ml of the inducer nisin. Both *tcdA* and *tcdB* transcript levels were significantly reduced in *C. difficile* overexpressing *dccA*, but not in the *dccA*^{mut} or vector controls, upon induction with nisin (Fig. 1A); the *tcdA* and *tcdB* transcript levels were reduced to 5% and 11%, respectively, of the transcript levels in the induced vector controls. The level of the *codY* transcript, previously shown not to be regulated by c-di-GMP, was not affected by *dccA* expression (see Fig. S2 in the supplemental material) (35). The level of c-di-GMP in *C. difficile* with pDccA grown with 1 μg/ml nisin is anticipated to be very high compared to basal levels; this concentration of nisin was previously determined to result in ~1,000-fold-increased intracellular c-di-GMP levels (35). Therefore, the effect of more subtle increases in c-di-GMP levels on toxin gene expression was assessed. To do this, the level of the *tcdA* transcript in *C. difficile* with pDccA, grown in BHIS-Tm medium with 0, 0.01, 0.1, or 1 μg/ml nisin, was measured. The *flgB* transcript was included as a control. As expected, the level of the *flgB* transcript decreased with increasing nisin concentrations (Fig. 1B). The *flgB* transcript level was significantly reduced in *C. difficile* with pDccA grown in 0.01 μg/ml nisin, which corresponds to ~2-fold-increased c-di-GMP levels (35), compared to the uninduced vector control (Fig. 1B). Similarly, transcript levels for *tcdA* were significantly reduced by 50% in *C. difficile* with pDccA grown in medium with 0.01 μg/ml nisin. The *tcdA* transcript level further decreased in a dose-dependent manner.

The expression of toxin genes by *C. difficile* is typically studied in the context of TY medium rather than BHIS medium, because the *tcdA* toxin gene expression level is considerably higher in TY medium (83). This is at least partly due to inhibition of toxin gene expression by glucose (21, 83), which is present at higher levels in BHIS than in TY medium. Therefore, we also examined the effect of c-di-GMP on toxin gene expression in *C. difficile* with pDccA or the vector during growth in TY medium. In TY medium, growth in the presence of nisin to early stationary phase resulted in an ~8-fold induction of *dccA* expression compared to the vector control (see Fig. S3 in the supplemental material). This resulted in corresponding 82%, 71%, and 93% decreases in the levels of the *flgB*, *sigD*, and *flgM* transcripts, respectively (see Fig. S3 in the supplemental material), indicating that increasing the intracellular c-di-GMP concentration represses at least some flagellar genes in *C. difficile* grown in TY medium as in BHIS medium. Also consistent with the regulation by c-di-GMP that we observed in BHIS broth, induction of *dccA* during growth in TY medium decreased the abundance of the *tcdA* and *tcdB* transcripts by 99% and 94%, respectively (see Fig. S3 in the supplemental material). We conclude based on these data that c-di-GMP represses several flagellar genes and the *tcdA* and *tcdB* toxin genes during growth in TY and BHIS media. Because phenotypes are readily observable in BHIS medium as in TY medium, all subsequent experiments were done with BHIS medium.

Next, the effects of c-di-GMP on toxin production were examined by Western blotting. TcdA toxin was assayed in cell lysates and supernatants from *C. difficile* with pDccA, pDccA^{mut}, or the vector grown in BHIS-Tm medium with or without 1 μg/ml nisin. TcdA protein was essentially undetectable in *C. difficile* overexpressing *dccA*, both in the cell lysates (data not shown) and in supernatants (Fig. 1C). Consistent with these results, supernatants from *C. difficile* overexpressing *dccA* were less cytopathic to cultured human fibroblasts than supernatants from *C. difficile* carrying the vector (Fig. 2). Whereas culture supernatants from *C. difficile* with the vector caused rounding of the fibroblasts, supernatants from *C. difficile* with pDccA did not. Figure 2A (top) shows the morphologies of fibroblasts treated with the indicated culture supernatants diluted 1:40 in tissue culture medium. As a measure of production of functional toxin by these strains, the titer of supernatant that caused 100% cell rounding (cytopathic effect [CPE]) was determined (Fig. 2B). A significantly reduced CPE on the fibroblasts was observed for *C. difficile* overexpressing *dccA* even in exponential-phase, when the level of toxin production is relatively low (83, 84), as well as in stationary-phase cul-

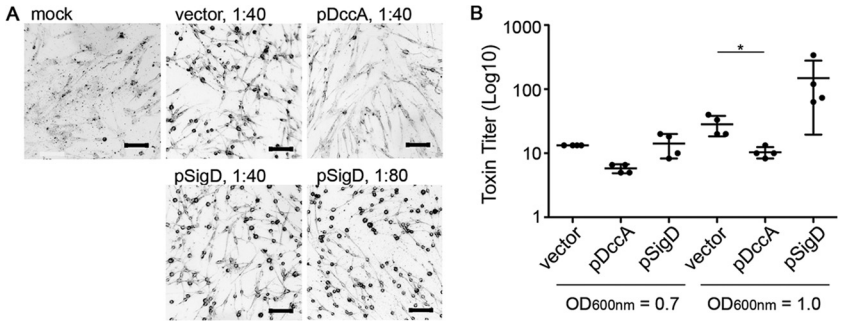

FIG 2 Cytopathic effect of *C. difficile* 630 overexpressing *dccA* or *sigD* on fibroblasts. Supernatants from *C. difficile* with the indicated plasmids, grown to exponential phase (OD at 600 nm of ~0.6 to 0.8) or early stationary phase (OD at 600 nm of ~1.0 to 1.2) in BHIS-Tm medium containing 1 μg/ml nisin, were applied onto monolayers of human foreskin fibroblasts. The data shown are from experiments using supernatants from early-stationary-phase cultures. (A) Representative images of intoxicated cells and untreated controls (mock), with the dilution factors indicated. Bars = 100 μm. (B) CPE was evaluated after 20 h by determining the titer at which a 100% cytopathic effect was observed. Each symbol represents the average of three measurements for each of four independent biological samples, and the bars indicate the means and standard deviations. *, $P < 0.05$ by the Mann-Whitney test.

tures. It should be noted that fibroblasts are particularly sensitive to the effects of toxin B (TcdB) (85, 86). Because increasing c-di-GMP concentrations have a more robust effect on *tcdA* expression, this assay may underestimate the effects of c-di-GMP on the total cytopathicity of *C. difficile* culture supernatants. Taken together, these results indicate that c-di-GMP reduces expression levels of the toxin genes, which leads to reduced toxin production by *C. difficile*.

c-di-GMP impacts the expression of *tcdR* but not of genes encoding other known regulators of the toxin genes. To begin to determine the mechanism by which c-di-GMP regulates the toxin genes, we examined the effect of elevated c-di-GMP levels on *tcdR*, *tcdC*, *codY*, and *sigH*, which are known or suspected regulators of *tcdA* and *tcdB* gene expression, by qRT-PCR as described above (11, 12, 17–19, 21, 22). Of these genes, only the level of the *tcdR* transcript was significantly reduced in *C. difficile* overexpressing *dccA* (Fig. 3). While the *tcdR* transcript level was 13-fold lower in *C. difficile* with elevated c-di-GMP concentrations than in the induced vector controls, *tcdC*, *codY*, and *sigH* transcripts were unaffected by c-di-GMP. These results indicate that c-di-GMP negatively regulates the expression of the *tcdR* gene, which provides a mechanism by which c-di-GMP could impact *tcdA* and *tcdB* expression. It remains possible that c-di-GMP affects the regulatory activity of TcdC, CodY, or SigH.

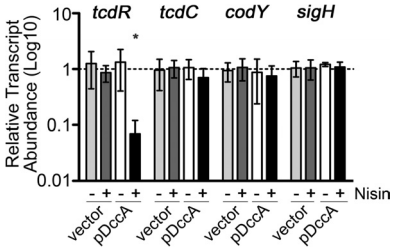

FIG 3 Regulation of known toxin gene regulators by c-di-GMP. The levels of the *tcdR*, *tcdC*, *codY*, and *sigH* transcripts, as indicated at the top, in *C. difficile* 630 with the vector or pDccA, grown to early stationary phase (OD at 600 nm of ~1.0 to 1.2) in BHIS-Tm medium with or without 1 μg/ml nisin, were measured by qRT-PCR. The data were analyzed as described in the legend of Fig. 1. *, $P < 0.05$ by one-way analysis of variance and Dunnett's multiple-comparison test comparing values to the average for the induced vector control.

The alternative sigma factor SigD positively regulates toxin gene expression. We further examined the possibility that co-regulation of flagellar genes and toxin genes could link toxin gene expression to c-di-GMP levels. The *sigD* gene, predicted to encode the flagellar alternative sigma factor, is among those in the *flgB* operon and is negatively regulated by c-di-GMP (35). Recently, it was reported that a mutation in *sigD* in *C. difficile* abrogates *tcdR*, *tcdA*, and *tcdB* expression (34). Moreover, the *sigD* transcript level was significantly reduced in several strains with mutations in the *flgB* operon, each of which also showed reduced toxin gene expression. Therefore, we hypothesized that SigD positively regulates *tcdR* expression and thus *tcdA* and *tcdB* expression. Repression of *sigD* by c-di-GMP consequently would be expected to reduce expression levels of the toxin genes.

This hypothesis leads to the following predictions: (i) expression of *sigD* should positively regulate *tcdR*, *tcdA*, and *tcdB* expression, and (ii) ectopic expression of *sigD* should unlink flagellar and toxin gene expression. To address the first prediction, because the *C. difficile sigD* mutant was previously described to have decreased toxin gene expression levels (34), we chose to instead overexpress *sigD* in *C. difficile* to determine whether this enhances toxin gene expression. The *sigD* gene was cloned into the expression vector pMC-Pcpr, allowing *sigD* expression to be controlled by the addition of nisin to the growth medium (35). *C. difficile* containing this expression plasmid, pSigD, or the empty vector was grown to exponential phase in BHIS-Tm medium in the presence or absence of 1 μg/ml nisin. At this phase of growth and in this growth medium, the toxin genes are typically expressed at very low levels (83, 84). Growth in the presence of nisin resulted in an ~30- to 50-fold increase in the *sigD* transcript level in *C. difficile* with pSigD but not the vector control, confirming that this method allows inducible expression of *sigD* (Fig. 4A, left). As evidence that a functional SigD was being produced, the transcript levels for *flgM*, *fliC*, and CD0241, chosen to represent the late-stage flagellar genes predicted to be positively regulated by SigD, were measured. The *flgM*, *fliC*, and CD0241 transcript levels were 9.9-, 4.4-, and 2.0-fold higher, respectively, in *C. difficile* with pSigD grown with nisin than in the vector control strain grown under the same conditions (Fig. 4A, right). Thus, in all three cases, these transcripts were significantly more abundant in *C. difficile* overexpressing *sigD*, albeit to various degrees, indicating that SigD

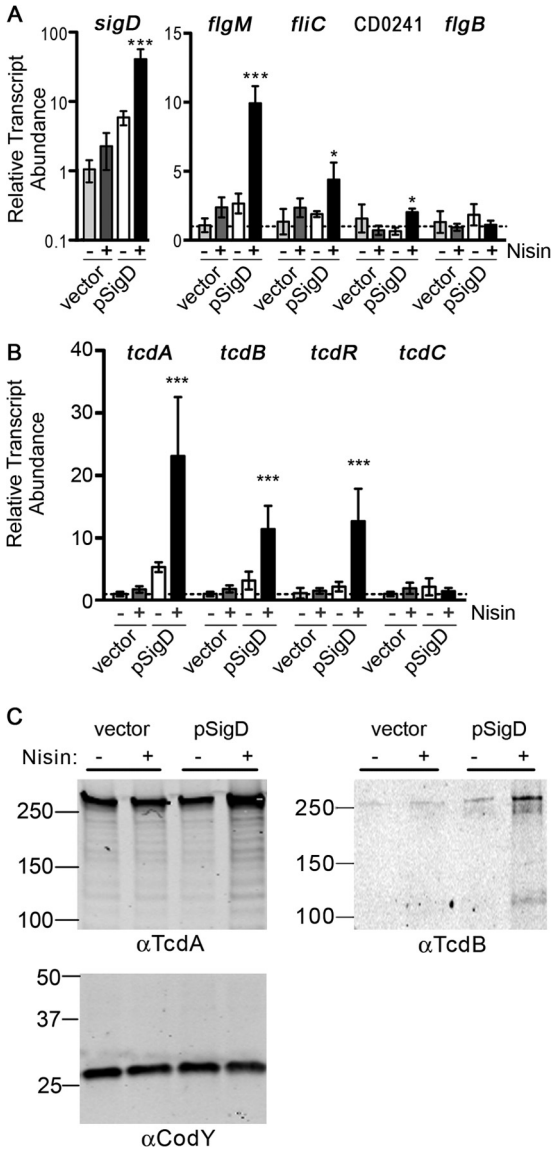

FIG 4 Overexpression of *sigD* results in increased flagellar and toxin gene expression in *C. difficile* 630. (A and B) *C. difficile* with pSigD or the vector was grown in BHIS-Tm medium with or without 1 μg/ml nisin to induce *sigD* expression. Samples were collected at exponential phase (OD at 600 nm of ~0.6 to 0.8), when the toxin gene expression level is relatively low. Transcript levels of the genes indicated at the top of the panels were assessed by qRT-PCR, as described in the text. (A) Effect of growth with nisin on *sigD* transcript abundance (left) and on the abundance of select flagellar gene transcripts (right). (B) Effect of *sigD* overexpression on toxin and regulator gene transcripts. Shown are the means and standard deviations from at least three independent samples. In panels A and B, the data for each transcript were analyzed by one-way analysis of variance and Dunnett's multiple-comparison test comparing values to the average for the induced vector control. *, $P < 0.05$; ***, $P < 0.001$. (C) Detection by Western blotting of TcdA, TcdB, and CodY in lysates of *C. difficile* 630 with the vector or pSigD, grown to early stationary phase (OD at 600 nm of ~1.0 to 1.2) in BHIS-Tm medium with or without 1 μg/ml nisin. TcdA and TcdB migrated more slowly than the highest-molecular-mass band in the protein marker (250 kDa), as expected due to their large size. CodY migrated at approximately 27 kDa, consistent with previous results (19).

is functional in *C. difficile*. The transcript level of *flgB* was not affected by the induction of *sigD* expression, indicating that there is no autoregulation of the *flgB* operon by SigD.

To determine the effect of SigD on toxin gene expression, we measured the levels of *tcdR*, *tcdA*, *tcdB*, and *tcdC* transcripts in *C. difficile* with the vector or pSigD, each grown with or without 1 μg/ml nisin. In *C. difficile* overexpressing *sigD*, the *tcdA*, *tcdB*, and *tcdR* transcript levels were increased approximately 22-, 10-, and 11-fold, respectively, while the *tcdC* transcript level remained unchanged (Fig. 4B). This expression pattern is consistent with and complementary to the previous observation that a mutation in *sigD* (and other mutations in the *flgB* operon) alters expression of *tcdR*, *tcdA*, and *tcdB* but not of *tcdC* (34) and our findings that c-di-GMP represses *tcdA*, *tcdB*, and *tcdR* but not *tcdC* (Fig. 1 and 3).

The effect of SigD on expression of the toxin genes was further demonstrated by Western blot and CPE assays. TcdA and TcdB proteins were more abundant in cell lysates of *C. difficile* overexpressing *sigD* but not in the control strains (Fig. 4C). As a control, we probed for the CodY protein and found it to be unaffected by overexpression of *sigD* (Fig. 4C). Interestingly, no difference was observed in TcdA or TcdB levels in supernatants from the same cultures (data not shown), suggesting that *sigD* expression does not substantially increase the export of the toxin proteins. Supernatants from stationary-phase cultures of *C. difficile* overexpressing *sigD* caused somewhat greater CPE on human foreskin fibroblasts than did supernatants from control cultures (Fig. 2A, bottom) but not significantly so (Fig. 2B). The level of induced CPE as a result of *sigD* expression was higher for supernatants from stationary-phase cultures than for supernatants from exponential-phase cultures. This result may reflect the accumulation of SigD as a consequence of longer induction times or the need for other growth phase-dependent regulators of toxin genes to coactivate expression. Together, these results support the hypothesis that SigD positively regulates toxin gene expression.

SigD induces toxin gene expression in the nonmotile *C. difficile* strain JIR8094. *C. difficile* JIR8094, an erythromycin-sensitive derivative of strain 630, contains an undefined mutation(s) that renders it nonmotile (see Fig. S4A in the supplemental material). In strain JIR8094, the *flgB*, *sigD*, *flgM*, and CD0241 transcripts were 50 to 100 times less abundant than in the 630 strain grown under the same conditions in parallel (see Fig. S4B in the supplemental material). We predicted that toxin gene expression would be reduced accordingly in strain JIR8094. Indeed, the level of the *tcdA* transcript was >100 times lower in strain JIR8094 than in the 630 parent strain (see Fig. S4B in the supplemental material). The *tcdB* and *tcdR* transcript levels were also significantly reduced in strain JIR8094, underscoring the tight link between flagellar and toxin gene expression in *C. difficile*.

To determine whether SigD can drive toxin gene expression in this strain that does not express flagellar genes, the pSigD expression plasmid and empty vector were introduced into *C. difficile* JIR8094. The resulting JIR8094 pSigD and vector control strains were grown in BHIS-Tm medium with or without 1 μg/ml nisin, and the levels of *sigD*, *flgM*, *tcdA*, *tcdB*, *tcdR*, and *tcdC* transcripts in these cells were measured. The addition of nisin to *C. difficile* JIR8094 with pSigD resulted in >100-fold-increased expression levels of *sigD* (Fig. 5). The *flgM* transcript was about 30-fold induced in these samples, indicating that ectopic expression of *sigD* can restore the expression of *flgM* in JIR8094. Upon induction of

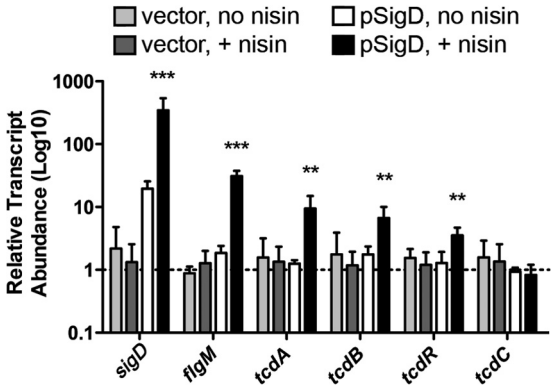

FIG 5 Overexpression of *sigD* partially restores toxin gene expression in nonmotile *C. difficile* JIR8094. *C. difficile* JIR8094 with pSigD or the vector was grown to mid-exponential phase (OD at 600 nm of ~0.6 to 0.8) in BHIS-Tm medium with or without 1 μg/ml nisin. Expression of the flagellar and toxin-related genes indicated at the top was assessed by qRT-PCR, as described in the text. Shown are the means and standard deviations from at least three independent samples. The data for each transcript were analyzed by one-way analysis of variance and Dunnett's multiple-comparison test comparing values to the average for the induced vector control. **, $P < 0.01$; ***, $P < 0.001$.

sigD expression in strain JIR8094, we observed significantly increased *tcdA*, *tcdB*, and *tcdR* transcript levels, which were 7-, 6-, and 3-fold more abundant, respectively, in JIR8094 expressing *sigD* than in the induced vector control (Fig. 5). As seen in the wild-type strain (Fig. 4), the *tcdC* transcript level was unchanged. We note that the fold changes in *tcdA*, *tcdB*, and *tcdR* transcript levels are comparable in 630 and JIR8094 expressing *sigD*, despite a greater induction of *sigD* expression in JIR8094 than in 630. This may signify that additional factors are missing or altered in JIR8094, preventing the full restoration of toxin gene expression with SigD.

Evidence for direct activation of *tcdR* expression by SigD. Our data show that expression of *sigD* results in increased expression levels of *tcdR*, *tcdA*, and *tcdB*, suggesting that the flagellar sigma factor SigD activates *tcdR* expression, and TcdR then upregulates *tcdA* and *tcdB* expression. However, this does not indicate whether SigD exclusively regulates the toxin genes through induction of *tcdR* expression or whether SigD can also upregulate *tcdA* and *tcdB* expression independent of TcdR. In addition, it is unknown whether SigD activates *tcdR* expression directly or whether SigD regulates another intermediate that in turn controls *tcdR*. We examined the relevant promoter regions for the presence of a SigD consensus binding sequence as an indication of direct binding by this sigma factor. The binding site for SigD in *C. difficile* has not been experimentally determined. The sequence may be similar to the FliA and SigD consensus binding sequences of *E. coli* and *B. subtilis*, respectively, because the consensus sequences are very similar to each other (49, 87) and because *B. subtilis* SigD can functionally complement a *fliA* mutation in *E. coli* and restore motility to the mutant (88). The consensus sequence of SigD from *E. coli* is TAAA-N_{15}-GCCGATAA, and the consensus sequence of the orthologous SigD protein from *B. subtilis* is CTAAA-N_{16}-CCGATAT (49, 87). Approximate matches to these sequences are present upstream of *flgM* (TAAA-N_{16}-GCGATAA) and *tcdR* (TATA-N_{15}-GCCGATTA). No matches were found upstream of *tcdA*, *tcdB*, CD0240, CD0241, or the flagellin gene *fliC*.

To determine whether SigD regulates the *tcdR* promoter directly and whether SigD can regulate the toxin gene promoters independent of TcdR, we took multiple approaches. First, we investigated whether ectopic expression of *sigD* results in increased toxin gene expression levels in a *tcdR* mutant as it does in wild-type *C. difficile*; the loss of upregulation of *tcdB* and *tcdB* transcripts upon *sigD* expression would indicate that SigD solely acts on *tcdR* expression, with indirect effects on the toxin. We used the Targetron method of gene inactivation to mutagenize *tcdR* in *C. difficile* 630ΔErm, yielding the *tcdR*::*ermB* strain (see Fig. S5A in the supplemental material). As expected based on previous studies showing that TcdR is a positive regulator of *tcdA* and *tcdB* expression (11, 12), the *C. difficile tcdR*::*ermB* mutant produced substantially less TcdA than did the 630ΔErm parent strain (see Fig. S5B in the supplemental material). The pSigD expression plasmid and the vector control were introduced into the *C. difficile tcdR*::*ermB* mutant by conjugation. The two strains were then grown in BHIS-Tm medium with or without 1 μg/ml nisin to mid-exponential phase; the *C. difficile* 630 strain carrying pSigD was included as a control to confirm the induction of *sigD* in response to nisin. We first determined the effect of *sigD* overexpression on toxin protein production in the *tcdR*::*ermB* mutant by Western blotting. Bacterial lysates from the mid-exponential-phase cultures were probed for TcdA, with CodY serving as a loading control (Fig. 6A, bottom). While an increased abundance of the TcdA protein was seen when *sigD* was overexpressed in the wild-type background, there was no discernible change in TcdA levels upon *sigD* expression in the *tcdR*::*ermB* background (Fig. 6A, top). Next, the effect of *sigD* expression on toxin gene expression in the *tcdR*::*ermB* mutant was assessed by qRT-PCR. In the *tcdR*::*ermB* mutant, as anticipated, *tcdA* and *tcdB* transcript levels were significantly reduced (96% and 93%, respectively) compared to those in the wild type (see Fig. S6 in the supplemental material). As observed in Fig. 4, *sigD* expression in the wild-type background led to increased *tcdA* and *tcdB* transcript levels (Fig. 6B). However, in the *tcdR*::*ermB* mutant, in which the induction of *sigD* expression was comparable (~4.5-fold), no significant differences in the *tcdA* or *tcdB* transcript levels were detected (Fig. 6C). Together, these results indicate that TcdR is required to mediate activation of *tcdA* and *tcdB* expression by SigD.

As an additional strategy, we coexpressed *sigD* along with a transcriptional fusion of *lacZ* to our promoters of interest in *B. subtilis*. The rationale for this experiment is that any *C. difficile*-specific factors that could mediate SigD activation of *C. difficile* toxin or flagellar promoters are likely to be absent in *B. subtilis*. Thus, increased reporter activity in response to SigD production would indicate direct SigD-mediated activation of the promoter in question. To do this, we began with *B. subtilis* strain MC202, in which the *C. difficile* two-component system *cprR-cprK* that senses and responds to nisin has been integrated at the *sacA* locus (78). Using strain MC190, which contains *cprR-cprK* as well as a *lacZ* fusion to the P_{cpr} promoter, used here to drive expression in response to nisin in *C. difficile* (78), we confirmed that these *B. subtilis* strains are capable of responding to nisin in a dose-dependent manner (data not shown). We inactivated the endogenous *sigD* gene by transformation of MC202 with genomic DNA from a previously described *B. subtilis sigD* mutant (50), generating strain RT838. We then introduced two additional fusions at the *amyE* locus: the P_{cpr}::*sigD* fusion, which allows nisin-inducible expression of *sigD*, and a *lacZ* fusion to the *tcdA*, *tcdB*, *tcdR*, or *flgM* promoter. The two fusions were transformed into RT838 on a

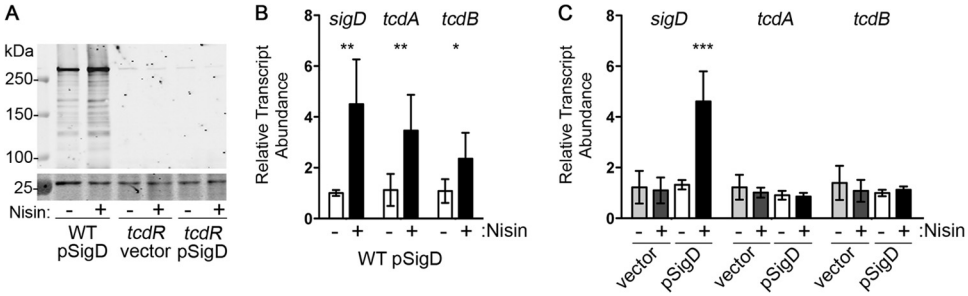

FIG 6 TcdR is required for SigD-mediated induction of toxin gene expression. *C. difficile* 630 with pSigD and the *C. difficile* 630ΔErm *tcdR*::*ermB* strain with the vector or with pSigD were grown to mid-exponential phase (OD at 600 nm of ~0.6 to 0.8) in BHIS-Tm medium with or without 1 μg/ml nisin. (A) The ability of SigD to induce toxin production in the absence of TcdR was assessed by probing for TcdA in bacterial lysates by Western blotting. The CodY protein was used as a loading control. Shown is a representative image from two independent experiments. WT, wild type. (B and C) The effect of *sigD* overexpression on toxin gene expression in the absence of TcdR was determined by qRT-PCR. (B) The *sigD*, *tcdA*, and *tcdB* transcripts in *C. difficile* 630 with pSigD were measured to confirm *sigD* induction and consequent effects on *tcdA* and *tcdB* transcript levels in response to growth with nisin. Shown are the means and standard deviations ($n = 5$). *, $P < 0.05$; **, $P < 0.01$ (determined by the Mann-Whitney test comparing values obtained with and those obtained without nisin for each transcript). (C) In parallel, the transcript levels of *sigD*, *tcdA*, and *tcdB* were determined for the *C. difficile* 630ΔErm *tcdR*::*ermB* strain with the vector or pSigD. Shown are the means and standard deviations ($n = 5$). ***, $P < 0.001$ by one-way analysis of variance and Dunnett's multiple-comparison test comparing values to the average for the induced vector control.

single plasmid in which the fusions are adjacent and divergently encoded. Thus, with the final strains, we used nisin to induce *sigD* expression and measured the effect of the resulting *C. difficile* SigD production on *tcdA*, *tcdB*, *tcdR*, and *flgM* promoter activity using β-galactosidase reporter assays.

The *B. subtilis* reporter strains were grown for 6 h at 37°C in LB medium containing 0, 0.5, 1.0, or 2.0 μg/ml nisin to induce *sigD* expression. The baseline β-galactosidase activity (i.e., activity detected in uninduced cultures) varied depending on the promoter, likely indicating differences in promoter strength in the *B. subtilis* host. The baseline activity for the P_{tcdR}::*lacZ* reporter grown in the absence of nisin was particularly low. The P_{flgM}::*lacZ* reporter strain, which we included as a positive control given our evidence that SigD positively regulates *flgM* expression, showed approximately 4- to 8-fold-higher β-galactosidase activity when grown in the presence of nisin (Fig. 7, left). Activation of P_{flgM}::*lacZ* activity was dose dependent to a degree, with 1 μg/ml nisin being sufficient for maximal expression. β-Galactosidase activity remained relatively unchanged in the P_{tcdA}::*lacZ* and P_{tcdB}::*lacZ* reporter strains regardless of the addition of nisin, with the exception of a small increase in activity in the P_{tcdB}::*lacZ* reporter strain at the highest nisin concentration (Fig. 7, right). In contrast, the P_{tcdR}::*lacZ* reporter showed a marked dose-dependent increase in β-galactosidase activity. In the presence of 2 μg/ml nisin, a 70-fold increase in activity was observed compared to that of the uninduced control. These data indicate that *C. difficile* SigD activates the *flgM* and *tcdR* promoters but not the *tcdA* or *tcdB* promoters. Moreover, because these experiments were performed with *B. subtilis*, and thus in the absence of other *C. difficile*-specific factors, the effect of SigD on *flgM* and *tcdR* is likely through direct binding to these promoters.

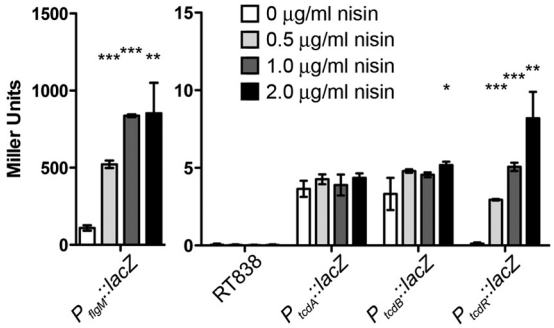

FIG 7 Effect of *C. difficile sigD* expression on *flgM*, *tcdR*, *tcdA*, and *tcdB* promoter activities. The *B. subtilis* reporter strains were grown in LB broth supplemented with nisin at 0, 0.5, 1.0, and 2.0 μg/ml to induce expression of *C. difficile sigD*. β-Galactosidase activity was measured to determine the activity of the reporter fusions indicated on the *x* axis. The assay was performed with three independent samples of each strain at each nisin concentration. Means and standard deviations are shown. The data were analyzed by an unpaired *t* test comparing induced cultures to the uninduced cultures for each strain. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

DISCUSSION

The two glucosylating toxins produced by *C. difficile*, TcdA and TcdB, play a central role in the development of disease symptoms in CDI (9, 10). There is great interest in understanding how these toxins are regulated, as this knowledge may lead to the identification of ways to inhibit toxin production to thereby reduce CDI. In this work, we show that the second messenger c-di-GMP represses the expression of the *C. difficile* toxin genes *tcdA* and *tcdB* as well as the expression of the *tcdR* gene. We also provide evidence that c-di-GMP regulation of *tcdR*, and thereby *tcdA* and *tcdB*, likely occurs through the control of expression of the alternative flagellar sigma factor gene *sigD*. Furthermore, our data suggest that SigD activates *tcdR* expression by interacting directly with the *tcdR* promoter and that SigD cannot activate *tcdA* and *tcdB* toxin gene expression independent of TcdR.

The ectopic expression of *sigD* in *C. difficile* was shown to increase the level of several flagellar gene transcripts, providing evidence that SigD is functional in *C. difficile*. Interestingly, *sigD* overexpression led to a 10-fold increase in the level of the *flgM* transcript, indicating that SigD regulates the expression of its own antagonist. This might be expected to neutralize the effects of *sigD* overexpression, but this was not observed. One possibility is that *C. difficile* encodes a nonfunctional FlgM; however, this has not been determined experimentally. Alternatively, the high level of SigD achieved through overexpression could exceed the amount

of FlgM in the cell. Thus, FlgM would effectively titrate a portion of available SigD, but sufficient SigD would remain in excess to activate other target promoters.

The data here reiterate that toxin gene expression is tied to the expression of flagellar genes. A link between flagellar and toxin gene expression was recently demonstrated (33, 34). The mechanism connecting the two was not addressed, although SigD was implicated in work by Aubry et al. (34). Specifically, it was shown that a *C. difficile sigD* mutant expresses the *tcdR*, *tcdA*, and *tcdB* toxin genes at dramatically lower levels, which is consistent with our finding that overexpression of *sigD* in *C. difficile* leads to increased *tcdR*, *tcdA*, and *tcdB* transcript levels. A likely scenario is that SigD activates the expression of *tcdR*, which in turn activates the expression of the toxin genes. This is supported by our finding that *sigD* overexpression in a *C. difficile tcdR* mutant does not lead to increased toxin gene expression or increased production of the TcdA protein. Furthermore, we provide evidence that SigD directly activates *tcdR* expression using a reporter system in a heterologous bacterial host, *B. subtilis*, in which *C. difficile*-specific regulatory factors are absent. The induction of *sigD* in this system led to the activation of the *flgM* and *tcdR* promoters but not the *tcdA* or *tcdB* promoters. Together, these data support the conclusion that upregulation of *tcdA* and *tcdB* in *C. difficile* by SigD occurs solely through TcdR. We attempted electrophoretic mobility shift assays using purified His_6-tagged SigD and promoters of interest to determine whether SigD interacts directly with the *tcdR* promoter. However, we were unable to purify SigD-His_6 in a sufficient amount or with sufficient purity to complete these experiments, and we could not detect a shift of the *flgM* promoter, which served as the positive control. Additional studies will be necessary to determine if and how SigD interacts with the *tcdR* promoter.

The connection between flagellar and toxin gene expression observed previously (34) is supported by the differences seen between *C. difficile* strain 630 and the nonmotile derivative JIR8094. Our data show that strain JIR8094 not only expresses flagellar genes at levels that are orders of magnitude lower than strain 630, JIR8094 also expresses toxin genes at significantly lower levels. This observation may help explain conflicting results regarding the relative importance of TcdA and TcdB toxins to *C. difficile* virulence. Kuehne et al. demonstrated previously that both toxins play important roles in virulence in hamsters by using the *C. difficile* 630ΔErm strain and *tcdA* and *tcdB* mutant derivatives (9). An earlier study showed that hamsters infected with *C. difficile tcdB* mutants, but not *tcdA* mutants, survived longer than hamsters infected with the parental strain, indicating that TcdB is the major virulence factor of *C. difficile* (10). However, the latter study used JIR8094 as the parental strain. The differences in outcomes of the studies by Lyras et al. and Kuehne et al. have been attributed to genetic differences between strains 630ΔErm and JIR8094, both of which were derived from strain 630 by serial passaging (89–91). Indeed, differences in toxin production between the two strains were reported previously, with strain 630ΔErm producing 3-fold more toxin than the JIR8094 derivative (91). These results are consistent with our findings that the *tcdA* transcript is 100-fold more abundant in strain 630 than in strain JIR8094. Thus, working with strain JIR8094 may have led to an underestimation of the importance of TcdA to virulence, as the relative difference in *tcdA* expression levels between strain JIR8094 and its *tcdA* mutant derivative would be smaller than that in the 630 background. Furthermore, because it is less toxigenic but clearly virulent, strain JIR8094 may be a good genetic background for studying other putative virulence factors in the hamster model, which is extremely sensitive to *C. difficile* toxins. Finally, it is interesting to speculate that so-called hypervirulent, epidemic strains that produce higher levels of toxin may do so because of increased flagellar gene, and in particular *sigD*, expression levels. This is supported by the observation that epidemic strain R20291 is more motile than strain 630 (92), although this was not reproduced by using a slightly different motility assay (35).

A precedent for the regulation of genes unrelated to motility and chemotaxis by SigD homologues exists for several bacterial species (93–97). For example, in the diarrheal pathogen *Vibrio cholerae*, the SigD orthologue FliA similarly augments the production of cholera toxin (98). This occurs as result of FliA repression of the quorum-sensing regulator HapR (a negative regulator of cholera toxin genes), leading to derepression of cholera toxin genes (98). It was recently demonstrated that in *C. difficile*, the Agr quorum-sensing system broadly impacts flagellar gene expression as well as the expression of *tcdA* (99). Regulation may occur through modulation of c-di-GMP, as the *agr* mutant also showed reduced expression levels of multiple genes encoding c-di-GMP hydrolytic enzymes. Quorum sensing and c-di-GMP signaling may thus work together to modulate flagellar and toxin gene expression in *C. difficile*. Finally, c-di-GMP and SigD may have broader effects on gene expression beyond controlling flagellar and toxin genes in *C. difficile*, so characterizing the c-di-GMP and SigD regulons is also of interest.

During growth in rich medium *in vitro*, flagellar and toxin genes appear to be expressed at distinct growth phases. *C. difficile* expresses flagellar genes at higher levels during exponential growth than during early stationary phase (22). Conversely, the production of the TcdA and TcdB toxins is negligible during exponential phase but is upregulated during stationary phase (83, 84, 100). Growth phase-dependent control of toxin production occurs at least in part through the activities of CodY and CcpA, which respond to nutrient depletion in the growth medium and derepress toxin genes during stationary phase and/or nutrient limitation (18–21). The *in vitro* data presented suggest that the effect of SigD expression on toxin gene expression is counteracted by the activities of CodY and/or CcpA during exponential growth but is unmasked during stationary phase as CodY and CcpA become inactive. However, it is unlikely that studies performed by using bacteria grown in rich medium, either TY (peptide-rich, low-glucose) or BHIS (peptide-rich, higher-glucose) medium, fully and accurately reflect the dynamics of flagellum and toxin regulation during infection, because glucose, amino acids, and peptides are scarce in the large bowel. During infection, regulation of toxin genes is likely more complex, involving an interplay between c-di-GMP, SigD, and other known or suspected regulators to precisely modulate toxin gene expression so that the bacteria produce toxin at the appropriate phase of infection. Nonetheless, SigD is clearly an important activator of toxin genes during infection, as evidenced by the dramatic attenuation of virulence in *C. difficile* flagellar mutants that produce less toxin (34) and the enhanced virulence of *fliC* and *fliD* mutants that produce more toxin (33). Future studies investigating the host signals impacting the level of intracellular c-di-GMP and the activities of other toxin gene regulators may elucidate the *in vivo* pathways controlling *C. difficile* virulence factor production.

The current study highlights the importance of c-di-GMP as a

regulator of *C. difficile* motility and toxin production. The working model of how c-di-GMP impacts toxin production, based on our and others' data, begins with c-di-GMP binding to the Cd1 riboswitch in the predicted 5′ untranslated region (UTR) of the *flgB* operon, which likely includes *sigD* (see Fig. S1 in the supplemental material), to inhibit its expression (45, 46). As a result, SigD will fail to activate expression of the late-stage flagellar operons, as we observed for *C. difficile* with high c-di-GMP levels. Because SigD also activates the expression of *tcdR*, c-di-GMP also represses toxin production. This model suggests that increases in c-di-GMP levels during the course of a *C. difficile* infection would cause reduced TcdA and TcdB production, possibly mitigating the virulence of the bacterium. Thus, components of the c-di-GMP signaling pathway, including the Cd1 riboswitch, are promising candidate targets for interfering with the ability of *C. difficile* to cause disease. Furthermore, determining the host-derived cues that impact bacterial intracellular c-di-GMP levels will inform us of the signals affecting virulence factor production of *C. difficile*.

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