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Journal Title: mBio
Volume: Volume 10, Number 2
Publisher: American Society for Microbiology: Open Access Journals | 2019-03-01
Type of Work: Article | Final Publisher PDF
Publisher DOI: 10.1128/mBio.01991-18
Permanent URL: https://pid.emory.edu/ark:/25593/tp70x

Final published version: http://dx.doi.org/10.1128/mBio.01991-18

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Accessed June 2, 2022 9:00 AM EDT
RstA Is a Major Regulator of *Clostridioides difficile* Toxin Production and Motility

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**ABSTRACT**  *Clostridioides difficile* infection (CDI) is a toxin-mediated diarrheal disease. Several factors have been identified that influence the production of the two major *C. difficile* toxins, TcdA and TcdB, but prior published evidence suggested that additional unknown factors were involved in toxin regulation. Previously, we identified a *C. difficile* regulator, RstA, that promotes sporulation and represses motility and toxin production. We observed that the predicted DNA-binding domain of RstA was required for RstA-dependent repression of toxin genes, motility genes, and *rstA* transcription. In this study, we further investigated the regulation of toxin and motility gene expression by RstA. DNA pulldown assays confirmed that RstA directly binds the *rstA* promoter via the predicted DNA-binding domain. Through mutational analysis of the *rstA* promoter, we identified several nucleotides that are important for RstA-dependent transcriptional regulation. Further, we observed that RstA directly binds and regulates the promoters of the toxin genes *tcdA* and *tcdB*, as well as the promoters for the *sigD* and *tcdR* genes, which encode regulators of toxin gene expression. Complementation analyses with the *Clostridium perfringens* RstA ortholog and a multispecies chimeric RstA protein revealed that the *C. difficile* C-terminal domain is required for RstA DNA-binding activity, suggesting that species-specific signaling controls RstA function. Our data demonstrate that RstA is a transcriptional repressor that autoregulates its own expression and directly inhibits transcription of the two toxin genes and two positive toxin regulators, thereby acting at multiple regulatory points to control toxin production.

**IMPORTANCE**  *Clostridioides difficile* is an anaerobic, gastrointestinal pathogen of humans and other mammals. *C. difficile* produces two major toxins, TcdA and TcdB, which cause the symptoms of the disease, and forms dormant endospores to survive the aerobic environment outside the host. A recently discovered regulatory factor, RstA, inhibits toxin production and positively influences spore formation. Herein, we determine that RstA directly binds its own promoter DNA to repress its own gene transcription. In addition, our data demonstrate that RstA directly represses toxin gene expression and gene expression of two toxin gene activators, TcdR and SigD, creating a complex regulatory network to tightly control toxin production. This study provides a novel regulatory link between *C. difficile* sporulation and toxin production. Further, our data suggest that *C. difficile* toxin production is regulated through a direct, species-specific sensing mechanism.

**KEYWORDS**  *Clostridium*, *Clostridium difficile*, RNPP, RRNPP, TcdA, TcdB, helix-turn-helix, motility, spore, sporulation, toxin, transcriptional regulator

*Clostridioides difficile* infection (CDI) is a nosocomial and community-acquired gastrointestinal disease that affects individuals with dysbiotic gut microbiota, which commonly occurs after antibiotic treatment (1, 2). Clinical outcomes range from mild diarrhea to severe disease symptoms, including sepsis and death (1). The two glyco-
sylating exotoxins, TcdA and TcdB, elicit CDI symptoms and are indispensable for C. difficile virulence (3). Environmental and intracellular signals, including nutrient availability and metabolic cues, strongly influence toxin production (4–7). There are numerous identified C. difficile factors that control toxin gene expression in response to these signals (8–12); however, the regulatory pathways and molecular mechanisms that directly control toxin gene expression are not fully understood (13).

Our previous work identified a novel regulator, RstA, which depresses C. difficile toxin production and motility (14). RstA inhibits transcription of the toxin genes tcdA and tcdB, the toxin-specific sigma factor, tcdR, and the flagellum-specific sigma factor, sigD, which is essential for motility and directs tcdR expression (11, 12, 14–16). In addition to repressing motility and toxin production, RstA positively influences C. difficile spore formation, which is critical for the survival of the bacterium outside of the host and for transmission from host to host, indicating that RstA regulates diverse phenotypes important for C. difficile pathogenesis. An rstA mutant exhibits increased toxin gene expression in vivo and is more virulent in the hamster model of CDI, demonstrating the impact RstA has on pathogenesis (14).

The predicted secondary structure of RstA reveals three apparent domains: an N-terminal conserved helix-turn-helix DNA-binding domain, followed by a series of multiple tetratricopeptide repeat (TPR) domains comprising a putative Spo0F-like protein-binding domain, and a C-terminal putative quorum-sensing-like domain (14). These characteristic features place RstA in the RRNPP (Rap/Rgg/NprR/PlcR/PrgX; former RNPP) family of proteins. RRNPP proteins are prevalent in Gram-positive organisms and regulate competence, sporulation, toxin production, and other important survival and virulence phenotypes (17–19). The DNA-binding or protein-binding activity of RRNPP proteins is controlled by the direct binding of small, quorum-sensing peptides (19). The precursor proteins encoding the quorum-sensing peptides are often adjacent to the regulatory RRNPP protein and are translated, exported, processed, and reinternalized at high cell densities (20–25). In addition, RRNPP proteins often auto-regulate their own expression, as is observed for RstA (14). The presence of these conserved domains within RstA provides insight into how RstA may regulate C. difficile toxin production, motility, and sporulation.

To better understand the regulatory impact RstA exerts on C. difficile toxin production and sporulation, we examined the function of the conserved DNA-binding domain. Our previous study (14) had shown that the DNA-binding domain is required for RstA-dependent regulation of rstA expression and toxin gene expression but is expendable for sporulation initiation. Here, we demonstrate that RstA directly binds to its promoter via an imperfect inverted repeat and that it directly binds the sigD and toxin gene promoters. Further, our data demonstrate that RstA and SigD independently control toxin expression, creating a multitiered regulatory pathway by which RstA represses toxin production. Finally, we show that the Clostridium perfringens rstA ortholog does not complement toxin production or sporulation in a C. difficile rstA mutant. However, a chimeric RstA protein containing the C. perfringens DNA-binding domain and the C. difficile Spo0F-binding and quorum-sensing-binding domains restores sporulation and represses toxin production, providing evidence that the ability to respond to species-specific signaling is necessary for RstA DNA-binding activity.

RESULTS

RstA autoregulates its gene transcription via an inverted repeat overlapping the promoter. Our previous work provided preliminary genetic evidence that the N-terminal putative helix-turn-helix DNA-binding domain was necessary for inhibition of toxin gene expression but was dispensable for sporulation initiation (14). However, further work with the recombinant His-tagged RstA proteins revealed that the constructs were expressed at low levels and were not detected by Western blotting of C. difficile lysates (data not shown). We created a new series of tagged proteins, possessing the 3×FLAG tag on the C-terminal end and found that these were stably expressed and easily detected in C. difficile rstA::erm lysates (see Fig. S2A in the supplemental
material). Corroborating our previous data (14), expression of the wild-type RstA, the full-length FLAG-tagged RstA, and the truncated RstAΔHTH-FLAG-tagged allele complemented sporulation in the rstA mutant (Fig. S2B). As previously observed (14), only full-length RstA restored toxin production to wild-type levels in the rstA background (Fig. S2C and D), confirming that the helix-turn-helix motif within the DNA-binding domain is essential for RstA-dependent control of toxin production.

We hypothesized that RstA directly binds to DNA to control toxin gene expression and transcription of additional target genes. This interaction is predicted to occur via the putative DNA-binding domain, as observed for other RRNPP transcriptional regulators (26–28). Additionally, we previously observed that rstA expression remains relatively unchanged throughout growth in multiple conditions and that rstA transcription is increased in an rstA mutant (14), suggesting that expression of rstA may be auto-regulated. To determine whether RstA is a DNA-binding protein, we first defined the rstA promoter region and probed the DNA-binding capability of RstA within its own promoter. The transcriptional start of rstA was identified at −32 bp upstream from the translational start using 5′ RACE. Corresponding σ^A−10 and −35 consensus sequences were detected immediately upstream of this transcriptional start site (Fig. 1A and B). To verify the mapped promoter and to determine whether any additional promoters are present that drive rstA transcription, a series of promoter fragments fused to the phoZ reporter gene was created, and alkaline phosphatase (AP) activity was measured in the 630Δerm reporter gene was created, and alkaline phosphatase (AP) activity was measured in the rstA-reporter construct on the phoZ background. P_{erm}::erm mutants. As previously observed, the full-length 489-bp rstA promoter fragment exhibited a 1.8-fold increase in activity in the rstA mutant compared to the parent strain, indicating RstA-dependent repression (Fig. 1C) (14). The truncated promoter fragments, PrstA_{291} and PrstA_{231}, produced similar fold changes in activity in the rstA mutant and parent strains, as observed for the full-length promoter. However, reporter activity was lower in the PrstA_{115} fragment compared to the longer fragments, suggesting that an enhancer sequence or an additional RstA-independent transcriptional activator is located between −231 bp to −115 bp upstream of the rstA open reading frame. A promoter fragment reporter fusion containing 380 bp of sequence upstream from the mapped rstA promoter (from −489 bp to −112 bp; intergenic region (IR) in Fig. 1A) was inactive, indicating that an additional promoter is not located within this region. We also tested whether RstA-dependent repression of the full-length PrstA reporter could be complemented. We expressed the rstA::FLAG construct from the nisin-inducible promoter, cprA (29), divergently from the PrstA::phoZ construct on the same plasmid in the rstA::erm background. PrstA reporter activity was reduced in a dose-dependent manner relative to the amount of nisin added to the medium (Fig. S3), further confirming the autoregulatory effect RstA exerts on its own expression. Altogether, the data demonstrate that the mapped σ^A-dependent promoter drives rstA expression and that RstA can repress transcription from this promoter.

The results obtained from the promoter-reporter fusions suggested that RstA binding was likely to occur within the 115 bp upstream of the translational start site. A 29-bp imperfect inverted repeat was identified within the predicted PrstA −10 consensus sequence, suggesting a possible regulatory binding site within this region (Fig. 1B). To determine whether this sequence serves as an RstA recognition site, we created a series of single nucleotide substitutions within the inverted repeat in the 489-bp PrstA reporter fusion, avoiding conserved residues required for RNAP-holoenzyme recognition (30). Most of the single nucleotide substitutions did not significantly alter reporter activity compared to the wild-type PrstA reporter (Fig. 1D). However, nucleotide substitutions in two positions, A-21 and T-19, abolished RstA repression in the parent strain, increasing reporter activity to match that of the rstA::erm mutant. These data suggest that the A-21 and T-19 nucleotides are important for RstA binding to the rstA promoter.

**RstA inhibits toxin and motility gene transcription.** Regulatory control of toxin gene expression in *C. difficile* involves multiple sigma factors and transcriptional regulators, which ensure that toxin production occurs in the appropriate environmental conditions (13). Our previous work (14) demonstrated that an rstA::erm mutant has
increased transcription of the *C. difficile* toxin genes, *tcdA* and *tcdB*, the toxin-specific sigma factor, *tcdR*, and the flagellum-specific sigma factor, *sigD*, which is required for motility and directs *tcdR* transcription (11, 12). To determine whether RstA is involved directly in repressing transcription of these genes, we first constructed *phoZ* reporter fusions with the promoter regions for each gene and examined RstA-dependent transcriptional activity.

The *tcdR* promoter region contains four identified independent promoter elements: a σ^A^-dependent promoter (−16 bp from the translational start), a σ^D^-dependent promoter (−76 bp from the translational start), and two putative σ^TcdR^-dependent promoters farther upstream (Fig. 2A) (11, 12, 31–33). Expression of the *tcdR* gene is relatively low in *C. difficile* (11, 32, 34), at least in part due to repression by CodY and CcpA binding throughout the *tcdR* promoter region under nutrient-rich conditions (8, 9, 33, 35, 36). We examined each of the promoter elements within P*tcdR* to determine whether RstA affects transcription from these promoters. A series of reporter fusions was created for each of the promoter elements, which were examined in the *rstA*::erm mutant and parent strain, and activity was measured after 24 h of growth in TY medium (Fig. 2A).
A full-length 517-bp P\textsubscript{tcdR}\textsubscript{::}phoZ reporter and the two $\sigma\textsuperscript{tcdR}$-dependent promoter fusions exhibited similar low reporter activities in the parent and \textit{rstA} strains (Fig. 2B). However, increased reporter activity was observed in the \textit{rstA} mutant for the individual and \textit{tcdR}-dependent promoter fusions. These results indicate that RstA impacts the function of these promoter elements and contributes to repression of \textit{tcdR} transcription.

![Diagram](image)

**FIG 2** RstA inhibits toxin gene expression. (A) A schematic of the promoter regions of \textit{tcdR}, \textit{tcdA}, and \textit{tcdB} denoting the relative locations of the transcriptional start sites experimentally demonstrated (12, 32–34) and the open reading frames of all three genes (not drawn to scale). Pale red boxes approximate CodY- and CcpA-binding sites within the toxin gene promoters (8, 9, 36). The yellow boxes indicate the locations and sizes of the promoter fragments constructed for the phoZ reporter fusions in panels B to D. Alkaline phosphatase (AP) activity of the P\textit{tcdR}\textsubscript{::}phoZ reporter fusions of various lengths (B) (promoterless phoZ [MC448], P\textit{tcdR}\textsubscript{A} [MC1285/MC1286], P\textit{tcdR}\textsubscript{D} [MC1145/MC1146], P\textit{tcdR}\textsubscript{P2} [MC1147/MC1148], and P\textit{tcdR}\textsubscript{P1} [MC1149/MC1150]) and the P\textit{tcdA}\textsubscript{::}phoZ (C) (−511 bp to −1 bp upstream of transcriptional start; MC1249/MC1250) or P\textit{tcdB}\textsubscript{::}phoZ (D) (−531 bp to −31 bp upstream of transcriptional start (MC1251/MC1252)) reporter fusions in strain 630Δerm and the \textit{rstA}\textsubscript{::}erm mutant (MC391) grown in TY medium (pH 7.4) at H\textsubscript{2}O\textsubscript{2}. The means and standard errors of the means for four biological replicates are shown. $^*$, $P \leq 0.05$, using Student’s $t$ test compared to the activity observed in the 630Δerm parent strain for each promoter construct.
strain, strain 630, and a current epidemic strain, strain R20291, are identical to the \( \alpha^A \) promoter sequence through the translational start site but diverge considerably upstream of this region (Fig. S4). No additional promoter elements were identified in the strain 630 or R20291 sequences upstream of the \( \alpha^A \)-dependent promoter (Fig. 3A). To determine whether RstA influences \( \text{sig}D \) transcription through repression of \( \text{PflgB} \), promoter reporter fusions representing each strain were constructed. As anticipated, activity of the strain 630Δ\( \text{erm} \) and R20291 \( \text{PflgB} \) reporters were higher in the \( \text{rstA} \) mutant than in the parent strain (1.7-fold and 1.5-fold, respectively; Fig. 3B), indicating that RstA represses \( \text{flgB} \) and consequently, \( \text{sigD} \) transcription.

RstA directly binds the \( \text{rstA}, \text{tcdR}, \text{flgB}, \text{tcdA}, \) and \( \text{tcdB} \) promoters via the conserved helix-turn-helix DNA-binding domain. To determine whether RstA directly binds target DNA, a variety of \textit{in vitro} electrophoretic gel shift assays were attempted, but no binding was observed in any condition tested. We considered that the lack of RstA-DNA interaction by gel shift may occur because of the absence of a cofactor, such as a quorum-sensing peptide, or because of a transient complex or oligomerization state. To overcome this obstacle, we performed biotin-labeled DNA pulldown assays to assess the DNA-binding capacity of RstA under native conditions. Biotinylated DNA was coupled to streptavidin beads as bait and incubated with cell lysates expressing either full-length RstA-FLAG or RstAΔHTH-FLAG protein. Specifically bound proteins were eluted and analyzed by Western blotting using FLAG M2 antibody.

We first tested the ability of RstA to directly interact with its own promoter. RstA-FLAG protein was recovered using the wild-type \( \text{rstA} \) promoter region as bait, demonstrating specific interaction of the RstA protein (Fig. 4A). However, the PrstA fragment did not capture RstAΔHTH-FLAG protein, indicating that the conserved HTH domain of RstA is essential for DNA binding. In addition to the wild-type \( \text{rstA} \) promoter, the PrstA T-19A and PrstA A-21C variants that eliminated RstA-dependent regulation \textit{in vivo} were used as bait (Fig. 1D). Both the PrstA T-19A and PrstA T-19A/A-21C variants captured significantly less RstA-FLAG than the wild-type promoter, suggesting that at least the T-19A nucleotide facilitates RstA interaction (Fig. 4A and Fig. S5A). The intergenic region upstream of the \( \text{rstA} \) promoter (Fig. 1A, IR) did not recover the full-length RstA-FLAG, indicating that RstA recognizes a specific DNA sequence within the promoter region. Finally, RstA-FLAG did not interact with unlabeled streptavidin beads nonspecifically (Fig. 4A and Fig. S5A). Altogether, these data demonstrate that RstA functions as a DNA-binding protein that directly and specifically binds its own promoter to repress transcription.
To determine whether RstA directly binds DNA to repress the transcription of genes encoding toxin regulators, we examined RstA binding to the flgB and tcdR promoter regions. RstA-FLAG protein bound specifically to the full-length tcdR promoter region, as well as the 630 and R20291 flgB promoters (Fig. 4B and Fig. S5B). Again, the HTH domain was required for these RstA-promoter interactions. To identify which internal promoter elements directly interact with RstA, previously characterized tcdR promoter fragments were used as bait (Fig. 2B), with the exception of a longer \( \alpha^{\text{TcdR}} \)-dependent (92-bp) fragment rather than 76-bp to limit potential steric hindrance of RstA binding due to the 5’ biotin label. This longer 92-bp Ptcdr(\( \alpha^A \)) fragment exhibited the same RstA-dependent regulation in reporter assays as the 76-bp reporter (Fig. S6). RstA-FLAG bound to the \( \alpha^A \)-dependent and \( \sigma^T \)-dependent tcdR promoter fragments but was not recovered from either of the \( \sigma^\text{tcdR} \)-dependent promoters (Fig. 4C and Fig. S5B), corroborating the reporter fusion results that demonstrated RstA repression of only the \( \alpha^A \)-dependent and \( \sigma^T \)-dependent tcdR promoter elements.

DNA pulldown assays were also performed to ascertain whether RstA directly binds to the tcdA and tcdB promoters. Both of the toxin promoters captured the full-length RstA-FLAG protein and failed to recover the RstA\( \Delta \)HTH-FLAG protein (Fig. 4D and Fig. S5B). These data provide direct biochemical evidence that RstA represses flgB, tcdR, tcdA, and tcdB transcription by binding to the promoter regions of these genes.

RstA represses toxin gene expression independently of SigD-mediated toxin regulation. Our data indicate that RstA represses toxin gene expression directly by binding to the tcdA and tcdB promoter regions and indirectly by repressing transcription of the sigma factors tcdR and sigD, which activate toxin gene expression. The biotin pulldown data suggest that RstA represses toxin gene expression through a multilayered regulatory pathway. To test whether direct repression of tcdA and tcdB transcription by RstA is physiologically relevant and independent of SigD, we created an rsta sigD double mutant and examined the impact of each mutation on toxin production. To aid in construction of an rsta sigD double mutant, we utilized the recently developed

**FIG 4** RstA binds to the rsta, tcdR, flgB, tcdA, and tcdB promoters. Western blot analysis using FLAG M2 antibody to detect recombinant RstA-3XFLAG or RstA\( \Delta \)HTH-3XFLAG in cell lysates or following biotin-labeled DNA pulldown assays. As a control, cell lysate expressing the RstA-3XFLAG construct (MC1004) or the RstA\( \Delta \)HTH-3XFLAG construct (MC1028) is included in the first lane or two of each Western blot shown. Additional negative controls in each panel include unbiotinylated full-length rsta promoter (\( - \)) and beads-only controls to ensure that RstA does not interact with the beads nonspecifically. The biotin-labeled fragments used as bait are of the 115-bp intergenic region upstream of the rsta promoter (IR; see Fig. 2; present in all panels) (A), the full-length tcdR (446-bp) or the 630\( \Delta \)erm or R20291 flgB (229-bp) promoters (B), the full-length tcdR (446-bp), \( \alpha^A \)-dependent (92-bp), \( \sigma^T \)-dependent (116-bp), \( \sigma^\text{tcdR} \)-dependent (188-bp), or \( \sigma^\text{sccp} \)-dependent (112-bp) promoters (C), or the full-length tcdR (446-bp), tcdA (511-bp), or tcdB (501-bp) promoters (D). All promoter fragments were bound to streptavidin-coated magnetic beads and incubated with C. difficile cell lysates grown in TY medium (pH 7.4) supplemented with 2 \( \mu \)g/ml thiamphenicol and 1 \( \mu \)g/ml nisin to mid-log phase (OD\( _{600} \) of 0.5 to 0.7), expressing either the RstA-3XFLAG construct (MC1004) or the RstA\( \Delta \)HTH-3XFLAG construct (MC1028).

To determine whether RstA directly binds DNA to repress the transcription of genes encoding toxin regulators, we examined RstA binding to the flgB and tcdR promoter regions. RstA-FLAG protein bound specifically to the full-length tcdR promoter region, as well as the 630 and R20291 flgB promoters (Fig. 4B and Fig. S5B). Again, the HTH domain was required for these RstA-promoter interactions. To identify which internal promoter elements directly interact with RstA, previously characterized tcdR promoter fragments were used as bait (Fig. 2B), with the exception of a longer \( \alpha^A \)-dependent promoter fragment (92 bp rather than 76 bp) to limit potential steric hindrance of RstA binding due to the 5’ biotin label. This longer 92-bp Ptcdr(\( \alpha^A \)) fragment exhibited the same RstA-dependent regulation in reporter assays as the 76-bp reporter (Fig. S6). RstA-FLAG bound to the \( \alpha^A \)-dependent and \( \sigma^T \)-dependent tcdR promoter fragments but was not recovered from either of the \( \sigma^\text{tcdR} \)-dependent promoters (Fig. 4C and Fig. S5B), corroborating the reporter fusion results that demonstrated RstA repression of only the \( \alpha^A \)-dependent and \( \sigma^T \)-dependent tcdR promoter elements.

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RstA represses toxin gene expression independently of SigD-mediated toxin regulation. Our data indicate that RstA represses toxin gene expression directly by binding to the tcdA and tcdB promoter regions and indirectly by repressing transcription of the sigma factors tcdR and sigD, which activate toxin gene expression. The biotin pulldown data suggest that RstA represses toxin gene expression through a multilayered regulatory pathway. To test whether direct repression of tcdA and tcdB transcription by RstA is physiologically relevant and independent of SigD, we created an rsta sigD double mutant and examined the impact of each mutation on toxin production. To aid in construction of an rsta sigD double mutant, we utilized the recently developed
CRISPR-Cas9 system modified for use in C. difficile to create an unmarked, nonpolar deletion of rstA in the 630Δerm and sigD::erm backgrounds (Fig. S7) (40). TcdA protein levels were ~3-fold higher in the rstA sigD double mutant than in the sigD mutant (Fig. 5A; total protein loaded shown in Fig. S8A), indicating that RstA represses toxin production independently of SigD. Overexpression of rstA in the rstA sigD mutant returned TcdA protein to the levels found in the sigD mutant. Likewise, a previously characterized sigD overexpression construct (11, 41) restored TcdA to wild-type levels in the rstA sigD mutant, further supporting that SigD and RstA regulate toxin production independently (Fig. 5A). In addition, transcript levels of tcdA, tcdB, and tcdR were increased in the rstA sigD mutant compared to the levels in the sigD mutant (Fig. 5B), mirroring the TcdA protein results. Altogether, these data provide further evidence that RstA is major regulator of toxin production that directly and indirectly represses toxin gene expression independently of SigD.

**RstA DNA-binding activity requires the species-specific C-terminal domains.**

The observation that RstA does not bind to target DNA in the tested *in vitro* conditions but does bind DNA in cell lysates suggests that a cofactor is required for RstA DNA-binding activity. We hypothesize that a small, quorum-sensing peptide serves as an activator for RstA DNA binding, as has been observed for other members of the RRNPP family (23–25, 42–44). To test this, we expressed RstA orthologs of other clostridial species (Fig. S9A) (45), including *Clostridium acetobutylicum*, *Clostridium perfringens*, and *Clostridium (Paeniclostridium) sordellii* in the *C. difficile* rstA mutant background. Only the *C. perfringens* RstA was stably produced in *C. difficile* (Fig. S9B). However, expression of the *C. perfringens* rstA ortholog failed to restore TcdA protein to wild-type levels (Fig. 6A; total protein loaded shown in Fig. S8B). *C. perfringens* RstA may be unable to repress *C.
difficile toxin production because the C. perfringens DNA-binding domain cannot recognize the C. difficile DNA target sequences and/or because the DNA-binding activity of C. perfringens RstA is not functional in C. difficile. To distinguish between these possibilities, we constructed a chimeric protein containing the C. perfringens DNA-binding domain (M1-Y51) fused to the C-terminal domains of the C. difficile RstA protein (herein known as CpHTH-CdCterminal3XFLAG) and examined the function of this chimeric RstA in the C. difficile rstA mutant. The RstA chimera restored C. difficile TcdA levels to those observed in the parent strain (Fig. 6A), indicating that the C. perfringens DNA-binding domain is functional in C. difficile. To confirm these results, we performed qRT-PCR analyses of tcdR, tcdA, and tcdB genes in these strains. The full-length C. perfringens RstA did not complement toxin gene expression in the C. difficile rstA mutant, while the CpHTH-CdCterminal3XFLAG chimeric RstA restored toxin gene transcript levels back to those observed in the parent strain (Fig. 6B), corroborating our previous results. These data strongly suggest that the C-terminal portion of RstA responds to species-specific signals to control the N-terminal DNA-binding activity.

Finally, we assessed the ability of a C. perfringens RstA to complement the low sporulation frequency of the C. difficile rstA mutant. Overexpressing the full-length C. perfringens RstA did not complement sporulation in the C. difficile rstA mutant (Fig. 6C). Unexpectedly, a hypersporulation phenotype was observed when the CpHTH-CdCterminal3XFLAG RstA chimera was expressed in the rstA mutant (Fig. 6C), indicating that the chimeric C. perfringens-C. difficile RstA promotes C. difficile sporulation to even higher levels than the native C. difficile RstA does. This hypersporulation phenotype

FIG 6 A hybrid rstA construct containing the C. perfringens DNA-binding domain with the C. difficile Spo0F-like and quorum-sensing-like domains complements C. difficile rstA toxin production and sporulation. (A) Western blot analysis of TcdA in 630Δerm pMC211 (MC282; vector control), rstA::erm pMC211 (MC505; vector control), rstA::erm pPcprA-rstA3XFLAG (MC1004), rstA::erm pPcprA-Cp-rstA3XFLAG (MC1324), and rstA::erm pPcprA-CpHTH2Cterminal3XFLAG (MC1257) grown in TY medium, pH 7.4, supplemented with 2 μg/ml thiamphenicol and 1 μg/ml nisin, at H24. The corresponding image showing total protein is shown in Fig. S8B. (B) qRT-PCR analysis of tcdR, tcdA, and tcdB transcript levels in 630Δerm pMC211 (MC282; vector control), rstA::erm pMC211 (MC505; vector control), rstA::erm pPcprA-rstA3XFLAG (MC1004), rstA::erm pPcprA-Cp-rstA3XFLAG (MC1324), and rstA::erm pPcprA-CpHTH2Cterminal3XFLAG (MC1257) grown in TY medium, pH 7.4, supplemented with 2 μg/ml thiamphenicol and 1 μg/ml nisin, at T3 (three hours after the entry into stationary phase). (C) Ethanol-resistant spore formation of 630Δerm pMC211 (MC282; vector control), rstA::erm pMC211 (MC505; vector control), rstA::erm pPcprA-rstA3XFLAG (MC1004), rstA::erm pPcprA-Cp-rstA3XFLAG (MC1324), and rstA::erm pPcprA-CpHTH2Cterminal3XFLAG (MC1257) grown on 70:30 sporulation agar supplemented with 2 μg/ml thiamphenicol and 1 μg/ml nisin. Sporulation frequency is calculated as the number of ethanol-resistant spores divided by the total number of cells enumerated at H24 as detailed in Materials and Methods. The means and standard errors of the means for at least three independent biological replicates are shown; asterisks represent P ≤ 0.05 by one-way ANOVA, followed by Dunnett’s multiple-comparison test compared to rstA pMC211 (MC505).
suggests that the *C. perfringens* HTH portion of the chimeric RstA protein alters the structure or activity of RstA to increase the positive effect on early sporulation events. These data warrant further investigation into the molecular mechanisms by which the C-terminal domains of RstA cooperate with the DNA-binding domain to promote sporulation.

**DISCUSSION**

The production of exotoxins and the ability to form quiescent endospores are two essential features of *C. difficile* pathogenesis. The regulatory links between toxin production and spore formation are complex and poorly understood. Some conserved sporulation regulatory factors, including Spo0A, CodY, and CcpA, strongly influence toxin production, yet some of these regulatory effects appear to be dependent on the strain or are indirect (8, 36, 46–48). Further, additional environmental conditions and metabolic signals, such as temperature and proline, glycine, and cysteine availability (5, 6, 10, 49), impact toxin production independently of these regulators, revealing the possibility that additional unknown factors are directly involved in toxin regulation (13). The recently discovered RRNPP regulator, RstA, represses toxin production and promotes spore formation, potentially providing a direct and inverse link between *C. difficile* spore formation and toxin biogenesis (14).

In this study, we show that RstA is a major, direct transcriptional regulator of *C. difficile* toxin gene expression. RstA inhibits toxin production by directly binding to the tcdA and tcdB promoters and repressing their transcription. RstA reinforces this repression by directly downregulating gene expression of tcdR, which encodes the sole sigma factor that drives tcdA and tcdB transcription. Finally, RstA directly represses the flgB promoter, inhibiting gene expression of the flagellum-specific sigma factor, SigD. SigD activates motility gene transcription but is also required for full expression of tcdR (11, 12). RstA repression of each major component in the toxin regulatory pathway creates a multitiered network in which RstA directly and indirectly controls tcdA and tcdB gene expression (Fig. 7).

RstA is the third characterized transcriptional repressor that directly binds to promoter regions for tcdR, tcdA, and tcdB, following two other transcriptional repressors, CodY and CcpA (8, 9, 36). The *in vivo* contribution of this reinforced repression of tcdA and tcdB transcription by CodY, CcpA, and RstA remains unknown. Interestingly, recent evidence has demonstrated that tcdR gene expression serves as a bistable switch that determines whether individual *C. difficile* cells within a population produce TcdA and TcdB, creating a divided population of toxin-OFF and toxin-ON cells (50). TcdR governs this bistability state by maintaining low basal expression levels, allowing for small changes to result in stochastic gene expression, and by positively regulating its own expression, establishing a positive-feedback loop that bolsters the toxin-ON state (50).
CodY was found to influence the population so that fewer cells produced toxin, but CcpA and RstA were not tested (50). We predict that both CcpA and RstA would bias the population of cells to a toxin-OFF state. Altogether, the tight control of tcdR transcription, reinforced by direct repression of tcdA and tcdB transcription by CcpA, CodY, and RstA, results in the convergence of multiple regulatory pathways at the bistable tcdR promoter to coordinate toxin production in response to nutritional and species-specific signals. This complex regulation ensures that the energy-intensive process of toxin production is initiated only to benefit the bacterium under the appropriate conditions.

Importantly, RstA is the first transcriptional regulator demonstrated to directly control flgB transcription initiation. To date, none of the previously identified regulators of flgB expression, including Spo0A, SigH, Agr, Hfq, SinR, and SinR, have been shown to bind promoter DNA and regulate flagellar gene expression through transcription initiation (46, 51–54). flgB expression is further regulated posttranscriptionally via a c-di-GMP riboswitch and a flagellar switch, both of which are located within the large, 496-bp 5’ untranslated region (39, 55, 56); however, the impact of RstA-mediated repression of flgB gene expression through additional pathways has not yet been explored.

Although we have identified several direct RstA targets, the sequence required to recruit RstA to target promoters remains unclear. The rstA promoter contains a near-perfect inverted repeat; however, this sequence is AT rich, as is the case for many C. difficile promoters. Imperfect inverted repeats were also found overlapping the −35 consensus sequences of the tcdA, tcdB, flgB, and α5-dependent tcdR promoters, and immediately upstream of the α5-dependent tcdR promoter (Fig. S10) (57), suggesting that RstA inhibits transcription at these promoters by sterically obstructing RNA polymerase docking. No clear consensus sequence defining an RstA box is delineated from these sequences. Other RRNPP regulators have also been found to bind imperfect, palindromic repeats or specific, conserved sequences in target promoters, but to our knowledge, only PlcR has a defined binding motif (24, 58, 59). Exhaustive attempts at ChIP-seq analysis to identify the C. difficile RstA regulon proved unsuccessful; however, our data implicate that RstA is a transcriptional repressor that directly controls multiple C. difficile phenotypes, and additional targets within the C. difficile genome seem likely.

The inability to recapitulate RstA-DNA binding with purified RstA in vitro together with the functional analysis of full-length and chimeric C. difficile and C. perfringens proteins suggest that (i) RstA DNA-binding activity requires a cofactor and (ii) this cofactor is species specific. Most RRNPP members are cotranscribed with their cognate quorum-sensing peptide precursor (19), but there are notable exceptions, including those encoded by unlinked genes (42, 60) and orphan receptors whose cognate ligands have not yet been discovered (61–63). RstA falls into this latter category, as there are no open reading frames adjacent to rstA that encode an apparent quorum-sensing peptide precursor. Importantly, no type of ligand other than small, quorum-sensing peptides has been identified for RRNPP proteins. In addition to RstA, other quorum-sensing factors have been implicated in C. difficile toxin production. The incomplete Agr1 and conserved Agr2 quorum-sensing systems induce toxin production through the production of a cyclic autoinducer peptide (AIP) that is sensed extracellularly (52, 64, 65); however, it is highly unlikely that the extracellular AIP molecule directly interacts with the cytosolic RstA protein. In addition, the interspecies LuxS-derived autoinducer-2 (AI-2) quorum-sensing molecule was found to increase C. difficile tcdA and tcdB gene expression, but not tcdR gene expression (66), indicating that AI-2 does not signal through RstA either. Identification of the cofactor that controls RstA activity is a high priority, as this will likely provide insight into the physiological conditions and/or metabolites that influence C. difficile TcdA and TcdB production.

Finally, as RstA is necessary for efficient C. difficile spore formation, the possibility remains that species-specific signaling is required for RstA-dependent control of early sporulation and that RstA coordinates C. difficile toxin production and spore formation in response to the same signal(s). Elucidating the molecular mechanisms that govern
| Plasmid or strain | Relevant genotype or feature(s) | Source, construction, or reference |
|------------------|--------------------------------|----------------------------------|
| **Plasmids**     |                                 |                                  |
| pRK24            | Tra<sup>+</sup> Mob<sup>+</sup> bla, tet | 78                               |
| pJK02            | E. coli-C. difficile shuttle vector; catP, cas9, pyrE sgRNA, pyrE homology region | 40                               |
| pMC123           | E. coli-C. difficile shuttle vector; bla catP | 29                               |
| pMC211           | pMC123 PcrpA                      | 77                               |
| pMC358           | pMC123 ::phoZ                     | 75                               |
| pMC67            | pMC123 PcrpA-rstA (CD3668)        | 14                               |
| pMC533           | pMC123 PcrpA-rstA (C. sordellii ATCC 9714) | This study                      |
| pMC543           | pMC123 Prst<sub>A</sub>phoZ       | 14                               |
| pMC559           | pMC123 Prst<sub>A</sub>27T:phoZ   | This study                       |
| pMC560           | pMC123 Prst<sub>A</sub>23A:phoZ   | This study                       |
| pMC561           | pMC123 Prst<sub>A</sub>19A:phoZ   | This study                       |
| pMC562           | pMC123 Prst<sub>A</sub>18I:phoZ   | This study                       |
| pMC563           | pMC123 Prst<sub>A</sub>17A:phoZ   | This study                       |
| pMC573           | pMC123 Prst<sub>A</sub>27C:phoZ   | This study                       |
| pMC574           | pMC123 Prst<sub>A</sub>24G:phoZ   | This study                       |
| pMC575           | pMC123 Prst<sub>A</sub>21C:phoZ   | This study                       |
| pMC576           | pMC123 Prst<sub>A</sub>19G:phoZ   | This study                       |
| pMC660           | pMC123 Prst<sub>A</sub>111:phoZ   | This study                       |
| pMC675           | pMC123 PcrpA-rstA-3XFLAG          | This study                       |
| pMC676           | pMC123 Prst<sub>A</sub>(380 bp):phoZ | This study                      |
| pMC677           | pMC123 Prst<sub>A</sub>23I:phoZ   | This study                       |
| pMC678           | pMC123 Prst<sub>A</sub>23I:phoZ   | This study                       |
| pMC682           | pMC123 PcrpA-rstA-HTH-3XFLAG       | This study                       |
| pMC713           | pMC123 PtdcR::phoZ                | This study                       |
| pMC726           | pJK02 with rasA homology region   | This study                       |
| pMC729           | pMC726 with rasA sgRNA (oMC1724)  | This study                       |
| pMC752           | pMC123 PtdcR(o<sup>A</sup>-92 bp)::phoZ | This study                   |
| pMC753           | pMC123 PtdcR(o<sup>B</sup>)::phoZ  | This study                       |
| pMC754           | pMC123 PtdcR(P2 o<sup>A</sup>cn::phoZ) | This study                   |
| pMC755           | pMC123 PtdcR(P1 o<sup>A</sup>cn::phoZ) | This study                   |
| pMC780           | pMC123 PcrpA-rstA (C. perfringens S13) | This study                   |
| pMC787           | pMC123 PcrpA-rstA (C. acetobutylicum ATCC 824) | This study                   |
| pMC795           | pMC123 PtdcA::phoZ                | This study                       |
| pMC796           | pMC123 PtdcB::phoZ                | This study                       |
| pMC798           | pMC123 PtdcA-rstA-HTHCdCterminal-3XFLAG | This study                   |
| pMC812           | pMC123 PtdcR(o<sup>A</sup>-76 bp)::phoZ | This study                   |
| pMC817           | pRT1824 Pfgb (630):phoZ           | This study                       |
| pMC818           | pRT1824 Pfgb (R20291):phoZ        | This study                       |
| pMC828           | pMC123 PcrpA-rstA-3XFLAG (C. acetobutylicum ATCC 824) | This study                   |
| pMC829           | pMC123 PcrpA-rstA-3XFLAG (C. perfringens S13) | This study                   |
| pMC830           | pMC123 PcrpA-rstA-3XFLAG (C. sordellii ATCC 9714) | This study                   |
| pMC888           | pMC123 Prst<sub>A</sub>phoZ PcrpA-rstA-3XFLAG | This study                   |
| pMC889           | pMC123 Prst<sub>A</sub>21A-19A::phoZ | This study                   |
| pRF144           | pMLT960 Pcw2-gusA                  | 79                              |
| pRT1824          | pMLT960 :phoZ                      | This study                       |
| pSigD            | pMC123 PcrpA-sigD                  | 11                              |

**E. coli strains**

HB101 pRK24 F<sup>−</sup> mcrB mrr hsdS20(rl− m<sub>K</sub>−) recA13 leuB6 ara-14 proA2 lacY1<sup>79</sup> galK2 xyl-5 mtl-1 rpsL20 pRK24

**C. difficile strains**

630<sup>Δerm</sup> Erm<sup>−</sup> derivative of strain 630  | Nigel Minton; 80 |
| 630<sup>Δerm</sup> pMC211                                 | 77                              |
| 630<sup>Δerm</sup> spo4<sup>A</sup>erm                    | 77                              |
| 630<sup>Δerm</sup> rstA<sup>A</sup>erm                    | 14                              |
| 630<sup>Δerm</sup> pMC358                                 | 75                              |
| 630<sup>Δerm</sup> rstA<sup>A</sup>erm pMC367              | 14                              |
| 630<sup>Δerm</sup> rstA<sup>A</sup>erm pMC211              | 14                              |
| 630<sup>Δerm</sup> sig<sup>A</sup>A<sup>+</sup>erm pMC211   | This study                      |
| 630<sup>Δerm</sup> rstA<sup>A</sup>erm pMC533              | This study                      |
| 630<sup>Δerm</sup> rstA<sup>A</sup>erm pMC543              | 14                              |

(Continued on next page)
| Plasmid or strain | Relevant genotype or feature(s) | Source, construction, or reference |
|------------------|--------------------------------|----------------------------------|
| MC830            | 630Δerm pMC559                | This study                       |
| MC831            | 630Δerm rstA::erm pMC559      | This study                       |
| MC832            | 630Δerm pMC560                | This study                       |
| MC833            | 630Δerm rstA::erm pMC560      | This study                       |
| MC834            | 630Δerm pMC561                | This study                       |
| MC835            | 630Δerm rstA::erm pMC561      | This study                       |
| MC836            | 630Δerm pMC562                | This study                       |
| MC837            | 630Δerm rstA::erm pMC562      | This study                       |
| MC838            | 630Δerm pMC563                | This study                       |
| MC839            | 630Δerm rstA::erm pMC563      | This study                       |
| MC840            | 630Δerm pMC564                | This study                       |
| MC856            | 630Δerm pMC573                | This study                       |
| MC857            | 630Δerm rstA::erm pMC573      | This study                       |
| MC858            | 630Δerm pMC574                | This study                       |
| MC859            | 630Δerm rstA::erm pMC574      | This study                       |
| MC860            | 630Δerm pMC575                | This study                       |
| MC861            | 630Δerm rstA::erm pMC575      | This study                       |
| MC862            | 630Δerm pMC576                | This study                       |
| MC863            | 630Δerm rstA::erm pMC576      | This study                       |
| MC879            | 630Δerm pMC660                | This study                       |
| MC880            | 630Δerm rstA::erm pMC660      | This study                       |
| MC1004           | 630Δerm rstA::erm pMC675      | This study                       |
| MC1008           | 630Δerm pMC676                | This study                       |
| MC1009           | 630Δerm rstA::erm pMC676      | This study                       |
| MC1010           | 630Δerm pMC677                | This study                       |
| MC1011           | 630Δerm rstA::erm pMC677      | This study                       |
| MC1012           | 630Δerm pMC678                | This study                       |
| MC1013           | 630Δerm rstA::erm pMC678      | This study                       |
| MC1028           | 630Δerm rstA::erm pMC682      | This study                       |
| MC1088           | 630Δerm pMC713                | This study                       |
| MC1089           | 630Δerm rstA::erm pMC713      | This study                       |
| MC1118           | 630Δerm ΔrstA                 | This study                       |
| MC1133           | 630Δerm pMC729                | This study                       |
| MC1143           | 630Δerm pMC752                | This study                       |
| MC1144           | 630Δerm rstA::erm pMC752      | This study                       |
| MC1145           | 630Δerm pMC753                | This study                       |
| MC1146           | 630Δerm rstA::erm pMC753      | This study                       |
| MC1147           | 630Δerm pMC754                | This study                       |
| MC1148           | 630Δerm rstA::erm pMC754      | This study                       |
| MC1149           | 630Δerm pMC755                | This study                       |
| MC1150           | 630Δerm rstA::erm pMC755      | This study                       |
| MC1193           | 630Δerm sigD::erm pMC729      | This study                       |
| MC1224           | 630Δerm ΔrstA pMC211          | This study                       |
| MC1225           | 630Δerm ΔrstA pMC367          | This study                       |
| MC1249           | 630Δerm pMC795                | This study                       |
| MC1250           | 630Δerm rstA::erm pMC795      | This study                       |
| MC1251           | 630Δerm pMC796                | This study                       |
| MC1252           | 630Δerm rstA::erm pMC796      | This study                       |
| MC1257           | 630Δerm rstA::erm pMC798      | This study                       |
| MC1278           | 630Δerm ΔrstA sigD::erm       | This study                       |
| MC1281           | 630Δerm ΔrstA sigD::erm pMC211| This study                       |
| MC1282           | 630Δerm ΔrstA sigD::erm pMC367| This study                       |
| MC1283           | 630Δerm ΔrstA sigD::erm pSigD | This study                       |
| MC1285           | 630Δerm pMC812                | This study                       |
| MC1286           | 630Δerm rstA::erm pMC812      | This study                       |
| MC1294           | 630Δerm pMC817                | This study                       |
| MC1295           | 630Δerm rstA::erm pMC817      | This study                       |
| MC1296           | 630Δerm pMC818                | This study                       |
| MC1297           | 630Δerm rstA::erm pMC818      | This study                       |
| MC1323           | 630Δerm rstA::erm pMC828      | This study                       |
| MC1324           | 630Δerm rstA::erm pMC829      | This study                       |
| MC1325           | 630Δerm rstA::erm pMC830      | This study                       |
| MC1433           | 630Δerm pMC889                | This study                       |
| MC1434           | 630Δerm rstA::erm pMC889      | This study                       |

(Continued on next page)
DXase I-treated RNA from the cultures were backdiluted into fresh TY medium supplemented with 5 \( \mu \)g/ml thiamphenicol and/or 1 \( \mu \)g/ml nisin throughout growth as needed (67). Overnight cultures of *C. difficile* were supplemented with 0.1% taurocholate and 0.2% fructose to promote sporule germination and prevent sporulation, respectively, as indicated (67, 68). *C. difficile* strains were cultured in a 37°C anaerobic chamber with an atmosphere of 10% \( H_2 \), 5% \( CO_2 \), and 85% \( N_2 \), as previously described (69). *Escherichia coli* strains were grown at 37°C in LB (70) with 100 \( \mu \)g/ml ampicillin and/or 20 \( \mu \)g/ml chloramphenicol as needed. Kanamycin (50 \( \mu \)g/ml) was used for counterselection against *E. coli* HB101 pRK24 after conjugation with *C. difficile*, as previously described (71).

**MATERIALS AND METHODS**

**Bacterial strains and growth conditions.** The bacterial strains and plasmids used in the study are listed in Table 1. **Clostridoides difficile** strains were routinely cultured in BHIS or TY medium (pH 7.4) supplemented with 2 to 5 \( \mu \)g/ml thiamphenicol and/or 1 \( \mu \)g/ml nisin throughout growth as needed (67). Overnight cultures of *C. difficile* were supplemented with 0.1% taurocholate and 0.2% fructose to promote spore germination and prevent sporulation, respectively, as indicated (67, 68). *C. difficile* strains were cultured in a 37°C anaerobic chamber with an atmosphere of 10% \( H_2 \), 5% \( CO_2 \), and 85% \( N_2 \), as previously described (69). *Escherichia coli* strains were grown at 37°C in LB (70) with 100 \( \mu \)g/ml ampicillin and/or 20 \( \mu \)g/ml chloramphenicol as needed. Kanamycin (50 \( \mu \)g/ml) was used for counterselection against *E. coli* HB101 pRK24 after conjugation with *C. difficile*, as previously described (71).

**Strain and plasmid construction and accession numbers.** Oligonucleotides used in this study are listed in Table 2. Details of vector construction are described in the supplemental material (see Fig. S1 in the supplemental material). *C. difficile* strains 630 (GenBank accession no. NC_009089.1) and R20291 (GenBank accession no. F545816.1), *Clostridium acetobutylicum* ATCC 824 (GenBank accession no. NC_003030.1), *Clostridium sordellii* ATCC 9714 (GenBank accession no. APW00000000), and *Clostridium perfringens* S13 (GenBank accession no. BA000016.3) were used as the templates for primer design and PCR amplification. The *rstA* ortholog from *C. acetobutylicum* was synthesized by Genscript (Piscataway, NJ). The *Streptococcus pyogenes* CRISPR-Cas9 system, which has been modified for use in *C. difficile* (40), was used to create a nonpolar deletion of the *rstA* gene. The 630\( \Delta \)erm and RT1075 (sigD:erm) strains containing the *rstA*-targeted CRISPR-Cas9 plasmid (MC1133 and MC1193, respectively) were grown overnight in TY medium with 5 \( \mu \)g/ml thiamphenicol. The next morning, the cultures were backdiluted into fresh TY medium supplemented with 5 \( \mu \)g/ml thiamphenicol and 100 ng/ml anhydrous tetracycline for 24 h to induce expression of the CRISPR-Cas9 system. A small aliquot of this culture was streaked onto BHIS plates, and colonies were screened by PCR for the presence or absence of the *rstA* allele.

**Mapping the *rstA* transcriptional start site (5' rapid amplification of cDNA ends (5' RACE).** DNase I-treated RNA from the *rstA*:erm mutant (MC391) was obtained as described above. 5' RACE was performed using the 5'/3' RACE kit, Second Generation (Roche), following the manufacturer’s instructions as previously reported (72). Briefly, first strand cDNA synthesis was performed using the *rstA*-specific primer oM982, followed by purification with the High Pure PCR Product purification kit (Roche). After subsequent poly(A) tailing of first strand cDNA, PCR amplification was performed using an oligo(T) primer and the *rstA*-specific primer oMC983 with Phusion DNA Polymerase (NEB). The resulting PCR products were purified from a 0.7% agarose gel (Qiagen) and TA cloned into pCR2.1 (Invitrogen) using the manufacturer’s supplied protocols. Plasmids were isolated and sequenced (Eurofins MWG Operon) to determine the transcriptional start site (~32 bp from translational start site; \( n = 7 \)).

**Spo0A activity will provide important insights into the regulatory control between sporulation and toxin production, reveal host cues and conditions that lead to increased toxin production, and help delineate the early sporulation events that control *C. difficile* Spo0A phosphorylation and activation.**

**TABLE 1**

| Plasmid or strain   | Relevant genotype or feature(s) | Source, construction, or reference |
|---------------------|---------------------------------|-----------------------------------|
| MC1435              | 630\( \Delta \)erm *rstA*:erm pMC888 | This study                       |
| RT1075              | 630\( \Delta \)erm sigD:erm       | 81                                 |

**Other strains**

| ATCC 824            | *Clostridium acetobutylicum*    | ATCC                               |
| ATCC 9714           | *Clostridium sordellii*         | ATCC                               |

RstA activity will provide important insights into the regulatory control between sporulation and toxin production, reveal host cues and conditions that lead to increased toxin production, and help delineate the early sporulation events that control *C. difficile* Spo0A phosphorylation and activation.
### TABLE 2 Oligonucleotides used in this studya

| Primer | Sequence (5'→3') | Use/locus tag/reference |
|--------|------------------|-------------------------|
| oMC44  | 5’CTAGCTGCTCTATGTTGCTACATC | Forward primer for rpoC qPCR (29) |
| oMC45  | 5’CCAGCTTCTCAGGATGCTACG | Reverse primer for rpoC qPCR (29) |
| oMC112 | 5’GGCAAATGGATTATTTGGAACATC | Forward primer for tcdB qPCR (77) |
| oMC113 | 5’TGGACTACATTTCTATCTCTGAC | Forward primer for tcdB qPCR (77) |
| oMC352 | 5’GGAGTAGGTGGTATGGTTTATATTAGGACG | Forward primer for confirmation of rtaA mutants |
| oMC547 | 5’TGGATAGTTGGAGAAGTCACTG | Forward primer for tcdA qPCR (77) |
| oMC548 | 5’GCTTGAATGTCCTAGTGGTGA | Forward primer for tcdA qPCR (77) |
| oMC891 | 5’GCCATGCGATCCAAAAGGTGGGAAATGATGGAAATTT | Forward primer for rtaA-3XFLAG constructs (14) |
| oMC982 | 5’TGGTCTTCAGGCTGTGTTAATGCTC | SP1 for rtaA 5’ RACE |
| oMC983 | 5’TGGTCTCTTGTGCTCTGTTATC | SP2 for rtaA 5’ RACE |
| oMC1006 | 5’GGAGCTTCCTCTTCTCTATCAGTTA | Reverse primer for checking sigD mutation |
| oMC1136 | 5’GGCGAAATTGGAATATAAGTGACTGATGGAAGCT | Forward primer for PrsA(A489) bp reporter fusion (14) |
| oMC1137 | 5’GCCGCGATCCACTATCCACCTCCTTTGGAACG | Reverse primer for PrsA reporter fusions (14) |
| oMC1145 | 5’ATTCCACAGTGCTCTTCTCCAAGCTCAAAATTCC | Forward SOE primer for rtaA-ΔHTH construct (14) |
| oMC1146 | 5’GCTTGGAGAAGGAACAGCTGTTGGAAATATCTGACC | Reverse SOE primer for rtaA-ΔHTH construct (14) |
| oMC1152 | 5’GCGATTGGATCTCTTAGGGGGGGGCAGCATG | Forward primer for C. sordelli ATCC 9714 rtaA (ATCC9714) | |
| oMC1153 | 5’GATGCGCTCGACTCCCCCTAAAAACTTAATCATTTATA | Reverse primer for C. sordelli ATCC 9714 rtaA (ATCC9714) | |
| oMC1204 | 5’TCCCAACACTTGAATGTTTTTCT | Reverse primer for checking rtaA mutants (14) |
| oMC1239 | 5’AAGTAGTTTTTTTTAAAATAATAAGTTA | A-27T mutation in rtaA promoter |
| oMC1240 | 5’TAACCTATTTATTTTTAAAAACACTCTT | A-27T mutation in rtaA promoter |
| oMC1241 | 5’AAGTAGTTTTTTTTAAAAATAATAAGTTA | T-23A mutation in rtaA promoter |
| oMC1242 | 5’TAACCTATTTATTTTTAAAAACACTCTT | T-23A mutation in rtaA promoter |
| oMC1243 | 5’AAGTAGTTTTTTTTAAAAATAATAAGTTA | T-19A mutation in rtaA promoter |
| oMC1244 | 5’TAACCTATTTATTTTTAAAAACACTCTT | T-19A mutation in rtaA promoter |
| oMC1245 | 5’AAGTAGTTTTTTTTAAAAATAATAAGTTA | T-19A mutation in rtaA promoter |
| oMC1246 | 5’TAACCTAATATTATTATTAAAAACACTCTT | A-18T mutation in rtaA promoter |
| oMC1247 | 5’AAGTAGTTTTTTTTAAAAATAATAAGTTA | A-18T mutation in rtaA promoter |
| oMC1248 | 5’TAACCTTATATTATTATTAAAAACACTCTT | T-17A mutation in rtaA promoter |
| oMC1325 | 5’AAGTAGTTTTTTTTAAAAATAATAAGTTA | T-17A mutation in rtaA promoter |
| oMC1326 | 5’TAACCTTATATTATTATTAAAAACACTCTT | T-17A mutation in rtaA promoter |
| oMC1327 | 5’AAGTAGTTTTTTTTAAAAATAATAAGTTA | A-27C mutation in rtaA promoter |
| oMC1328 | 5’TAACCTTATATTATTATTAAAAACACTCTT | A-27C mutation in rtaA promoter |
| oMC1329 | 5’AAGTAGTTTTTTTTAAAAATAATAAGTTA | A-24G mutation in rtaA promoter |
| oMC1330 | 5’TAACCTTATATTATTATTAAAAACACTCTT | A-24G mutation in rtaA promoter |
| oMC1331 | 5’AAGTAGTTTTTTTTAAAAATAATAAGTTA | A-21C mutation in rtaA promoter |
| oMC1332 | 5’TAACCTTATATTATTATTAAAAACACTCTT | A-21C mutation in rtaA promoter |
| oMC1527 | 5’GGGAAATTTACTATTGAGCTACAGTGAATAT | Forward primer for PrsA15; biotinylated |
| oMC1528 | 5’CATACATTCCACACTTCTTGAAG | Reverse primer for PrsA15 reporter fusion |
| oMC1529 | 5’GTCAGAATTTGCCCAATTGTTTTTGAAT | Forward primer for PrsA15 reporter fusion |

(Continued on next page)
| Primer | Sequence (5'→3') | Use/focus tag/reference |
|--------|------------------|------------------------|
| oMC1546 | 5'TGACCCTGATACGTGTTGAATAGCACGCTGATCAGGAAATTCG  |
| oMC1548 | 5'ATCTGATAGCCTGCTTCTTGTAGTATCCGATCTGACCTGATTCAAGT  |
| oMC1549 | 5'TGATATTGCTTCTTTCTGATGATGATGTGAGTATGATTCAAGT  |
| oMC1550 | 5'ATCTGATAGCCTGCTTCTTGTAGTATCCGATCTGACCTGATTCAAGT  |
| oMC1611 | 5'ATCTGATAGCCTGCTTCTTGTAGTATCCGATCTGACCTGATTCAAGT  |
| oMC1614 | 5'ATCTGATAGCCTGCTTCTTGTAGTATCCGATCTGACCTGATTCAAGT  |
| oMC1645 | 5'ATCTGATAGCCTGCTTCTTGTAGTATCCGATCTGACCTGATTCAAGT  |
| oMC1646 | 5'ATCTGATAGCCTGCTTCTTGTAGTATCCGATCTGACCTGATTCAAGT  |
| oMC1693 | 5'ATCTGATAGCCTGCTTCTTGTAGTATCCGATCTGACCTGATTCAAGT  |
| oMC1694 | 5'ATCTGATAGCCTGCTTCTTGTAGTATCCGATCTGACCTGATTCAAGT  |
| oMC1695 | 5'ATCTGATAGCCTGCTTCTTGTAGTATCCGATCTGACCTGATTCAAGT  |
| oMC1724 | 5'ATCTGATAGCCTGCTTCTTGTAGTATCCGATCTGACCTGATTCAAGT  |
| oMC1725 | 5'ATCTGATAGCCTGCTTCTTGTAGTATCCGATCTGACCTGATTCAAGT  |
| oMC1726 | 5'ATCTGATAGCCTGCTTCTTGTAGTATCCGATCTGACCTGATTCAAGT  |
| oMC1727 | 5'ATCTGATAGCCTGCTTCTTGTAGTATCCGATCTGACCTGATTCAAGT  |
| oMC1728 | 5'ATCTGATAGCCTGCTTCTTGTAGTATCCGATCTGACCTGATTCAAGT  |
| oMC1733 | 5'ATCTGATAGCCTGCTTCTTGTAGTATCCGATCTGACCTGATTCAAGT  |
| oMC1734 | 5'ATCTGATAGCCTGCTTCTTGTAGTATCCGATCTGACCTGATTCAAGT  |
| oMC1753 | 5'ATCTGATAGCCTGCTTCTTGTAGTATCCGATCTGACCTGATTCAAGT  |
| oMC1762 | 5'ATCTGATAGCCTGCTTCTTGTAGTATCCGATCTGACCTGATTCAAGT  |
| oMC1763 | 5'ATCTGATAGCCTGCTTCTTGTAGTATCCGATCTGACCTGATTCAAGT  |
| oMC1764 | 5'ATCTGATAGCCTGCTTCTTGTAGTATCCGATCTGACCTGATTCAAGT  |
| oMC1765 | 5'ATCTGATAGCCTGCTTCTTGTAGTATCCGATCTGACCTGATTCAAGT  |
| oMC1766 | 5'ATCTGATAGCCTGCTTCTTGTAGTATCCGATCTGACCTGATTCAAGT  |
| oMC1767 | 5'ATCTGATAGCCTGCTTCTTGTAGTATCCGATCTGACCTGATTCAAGT  |
| oMC1768 | 5'ATCTGATAGCCTGCTTCTTGTAGTATCCGATCTGACCTGATTCAAGT  |
| oMC1769 | 5'ATCTGATAGCCTGCTTCTTGTAGTATCCGATCTGACCTGATTCAAGT  |
| oMC1770 | 5'ATCTGATAGCCTGCTTCTTGTAGTATCCGATCTGACCTGATTCAAGT  |
| oMC1771 | 5'ATCTGATAGCCTGCTTCTTGTAGTATCCGATCTGACCTGATTCAAGT  |
| oMC1772 | 5'ATCTGATAGCCTGCTTCTTGTAGTATCCGATCTGACCTGATTCAAGT  |
| oMC1773 | 5'ATCTGATAGCCTGCTTCTTGTAGTATCCGATCTGACCTGATTCAAGT  |
| oMC1774 | 5'ATCTGATAGCCTGCTTCTTGTAGTATCCGATCTGACCTGATTCAAGT  |
| oMC1775 | 5'ATCTGATAGCCTGCTTCTTGTAGTATCCGATCTGACCTGATTCAAGT  |
| oMC1776 | 5'ATCTGATAGCCTGCTTCTTGTAGTATCCGATCTGACCTGATTCAAGT  |
| oMC1777 | 5'ATCTGATAGCCTGCTTCTTGTAGTATCCGATCTGACCTGATTCAAGT  |
| oMC1778 | 5'ATCTGATAGCCTGCTTCTTGTAGTATCCGATCTGACCTGATTCAAGT  |
| oMC1779 | 5'ATCTGATAGCCTGCTTCTTGTAGTATCCGATCTGACCTGATTCAAGT  |
| oMC1780 | 5'ATCTGATAGCCTGCTTCTTGTAGTATCCGATCTGACCTGATTCAAGT  |
| oMC1781 | 5'ATCTGATAGCCTGCTTCTTGTAGTATCCGATCTGACCTGATTCAAGT  |
| oMC1782 | 5'ATCTGATAGCCTGCTTCTTGTAGTATCCGATCTGACCTGATTCAAGT  |
| oMC1914 | 5'ATCTGATAGCCTGCTTCTTGTAGTATCCGATCTGACCTGATTCAAGT  |
| oMC1915 | 5'ATCTGATAGCCTGCTTCTTGTAGTATCCGATCTGACCTGATTCAAGT  |
| oMC1916 | 5'ATCTGATAGCCTGCTTCTTGTAGTATCCGATCTGACCTGATTCAAGT  |

(Continued on next page)
followed by Dunnett’s multiple-comparison test (GraphPad Prism v6.0), to compare sporulation efficiency to that of the rstA mutant.

**Alkaline phosphatase activity assays.** *C. difficile* strains containing the reporter fusions listed in Table 1 were grown and harvested on either 70:30 sporulation agar at H8, defined as eight hours after the cultures are applied to the plates (early stationary phase), or from TY liquid medium in stationary phase (T3, defined as three hours after the start of transition phase [approximately equivalent to H8 on plates; early stationary phase], or H24, defined as 24 h after the cultures are inoculated [late stationary phase]). Alkaline phosphatase assays were performed as described previously (75) with the exception that no chloroform was used for cell lysis. Technical duplicates were averaged, and the results are
presented as the means and standard errors of the means for three biological replicates. The two-tailed Student's *t* test was used to compare the activity in the parent strain.

**Biotin pulldown assays.** Biotin pulldown assays were performed as described by Jutras et al. (76). Briefly, a threefold excess of biotin-labeled DNA bait (30 μg) was coupled to streptavidin-coated magnetic beads (Invitrogen; binding capacity of 10 μg) in B/W buffer, and the bead-DNA complexes were washed with TE buffer to remove unbound DNA. In addition, an unbiotinylated PrstA (30 μg) negative control and a beads-only (dH2O) negative control were treated alongside the test DNA fragments to ensure that RstA did not interact nonspecifically with the streptavidin-coated magnetic beads. To determine the total amount of biotinylated-DNA bound to each bead preparation, each incubation and subsequent washes were quantitated via a Nanodrop 1000 and subtracted from the initial amount of DNA. To prepare cell lysates, *C. difficile* expressing either RstA-FLAG (MC1004) or RstAΔHTH-FLAG (MC1028) in the rstA background were grown to mid-log phase (OD600 of 0.5 to 0.7) in 500 ml TY medium (pH 7.4) supplemented with 2 μg/ml thiamphenicol and 1 μg/ml nisin, pelleted, rinsed with sterile water, and stored at −80°C overnight. The pellets were suspended in 4.5 ml BS/THES buffer and lysed by cycling between a dry ice/ethanol bath and a 37°C water bath. The cell lysates were vortexed for 1 min to shear genomic DNA, and cell debris was pelleted at 15K rpm for 15 min at 4°C. The supernatant, along with 10 μg salmon sperm DNA as a nonspecific competitor, was then applied to the bead-DNA complexes and rotated for 30 min at room temperature. This incubation was repeated once with additional supernatant and 10 μg salmon sperm DNA for two total incubations. The bead-DNA-protein complexes were washed seven times with BS/THES buffer supplemented with 10 μg/ml salmon sperm DNA and then without salmon sperm DNA to remove nonspecific proteins. The beads were transferred to clean microcentrifuge tubes twice during the washes to eliminate carry-over contamination. The remaining bound protein was eluted with 250 mM NaCl in Tris-HCl, pH 7.4, and the eluates were immediately analyzed by SDS-PAGE and Western blotting using FLAG M2 antibody (Sigma; see below). Each DNA bait fragment was tested in at least three independent experiments. As a control following each experiment, bait DNA was recovered by incubating the labeled beads in dH2O at 70°C for 10 min and analyzed on a 1.5% agarose gel to ensure that no cross-contamination occurred (data not shown). Densitometry was performed using Image Lab Software (Bio-Rad), and subsequent statistical analyses included a one-way ANOVA, followed by Dunnett’s multiple-comparison test (GraphPad Prism v6.0).

**Western blot analysis.** The indicated *C. difficile* strains were grown in TY medium (pH 7.4) supplemented with 2 μg/ml thiamphenicol and 1 μg/ml nisin at 37°C and harvested at H24 (24 h) (74). Total protein was quantitated using the Pierce Micro BCA protein assay kit (Thermo Scientific), and 8 μg of total protein was separated by electrophoresis on a precast 4 to 15% TGX stain-free gradient gel (Bio-Rad), and total protein was imaged using a ChemiDoc (Bio-Rad). Corresponding gel images for each Western blot are included in the supplemental material as indicated in the text. Protein was then transferred to a 0.45-μm nitrocellulose membrane, and Western blot analysis was conducted with either mouse anti-TcdA (Novus Biologicals) or mouse anti-FLAG (Sigma) primary antibody, followed by goat anti-mouse Alexa Fluor 488 (Life Technologies) secondary antibody. Imaging and densitometry were performed with a ChemiDoc and Image Lab software (Bio-Rad), and a one-way ANOVA, followed by Dunnett’s multiple-comparison test, was performed to assess statistical differences in TcdA protein levels between the rstA mutant and each rstA overexpression strain (GraphPad Prism v6.0). At least three biological replicates were analyzed for each strain, and a representative Western blot image is shown.

**Quantitative reverse transcription-PCR analysis.** *C. difficile* was cultivated in TY medium (pH 7.4) supplemented with 2 μg/ml thiamphenicol and 1 μg/ml nisin and harvested at T9 (defined as three hours after the start of transition phase; OD600 of 1.0 [approximately equivalent to H9 on plates]). Aliquots (3 ml) of culture were immediately mixed with 3 ml of ice-cold ethanol-acetone (1:1) and stored at −80°C. RNA was purified and DNase I treated (Ambion) as previously described (29, 35, 77), and cDNA was synthesized using random hexamers (77). Quantitative reverse transcription-PCR (qRT-PCR) analysis, using 50 ng cDNA per reaction and the SensiFAST SYBR & Fluorescein kit (Bioline), was performed in technical triplicates on a Roche Lightcycler 96. cDNA synthesis reaction mixtures containing no reverse transcriptase were included as a negative control to ensure that no genomic DNA contamination was present. Results are presented as the means and standard errors of the means for three biological replicates. Statistical significance was determined using a one-way ANOVA, followed by Dunnett’s multiple-comparison test (GraphPad Prism v6.0), to compare transcript levels between the rstA mutant and each rstA overexpression strain.

**SUPPLEMENTAL MATERIAL**

Supplemental material for this article may be found at https://doi.org/10.1128/mBio.01991-18.

FIG S1, PDF file, 0.1 MB.

FIG S2, PDF file, 1.3 MB.

FIG S3, PDF file, 0.6 MB.

FIG S4, PDF file, 0.8 MB.

FIG S5, PDF file, 0.7 MB.

FIG S6, PDF file, 0.5 MB.

FIG S7, PDF file, 0.9 MB.

FIG S8, PDF file, 1.1 MB.
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