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Multiple factors contribute to bimodal toxin gene expression in *Clostridioides* (*Clostridium*) *difficile*

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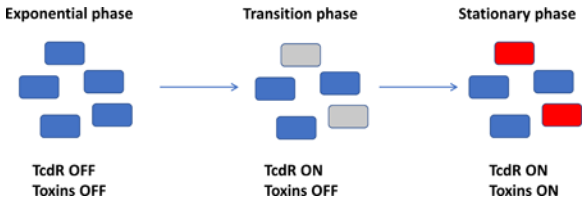
Abstract

Clostridioides (formerly *Clostridium*) *difficile* produces two major toxins, TcdA and TcdB, upon entry into stationary phase. Transcription of *tcdA* and *tcdB* requires the specialized sigma factor, σ^{TcdR}, which also directs RNA Polymerase to transcribe *tcdR* itself. We fused a gene for a red fluorescent protein to the *tcdA* promoter to study toxin gene expression at the level of individual *C. difficile* cells. Surprisingly, only a subset of cells became red fluorescent upon entry into stationary phase. Breaking the positive feedback loop that controls σ^{TcdR} production by engineering cells to express *tcdR* from a tetracycline-inducible promoter resulted in uniform fluorescence across the population. Experiments with two regulators of *tcdR* expression, σ^{D} and CodY, revealed neither is required for bimodal toxin gene expression. However, σ^{D} biased cells towards the Toxin-ON state, while CodY biased cells towards the Toxin-OFF state. Finally, toxin gene expression was observed in sporulating cells. We conclude that (i) toxin production is regulated by a bistable switch governed by σ^{TcdR}, which only accumulates to high enough levels to trigger toxin gene expression in a subset of cells, and (ii) toxin production and sporulation are not mutually exclusive developmental programs.

Graphical Abstract



Abbreviated Summary

Clostridioides difficile, the leading cause of antibiotic-associated diarrhea, produces toxins that inactivate host Rho GTPases. We used a fluorescent reporter to visualize toxin gene expression in

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Author contributions

EMR and GMK contributed to the design, execution, and interpretation of experiments. PMT contributed to the execution and interpretation of experiments. CDE and DSW contributed to the design and interpretation of the experiments. All authors contributed to the writing of the manuscript.

C. difficile within individual cells. Our findings imply toxin production is an example of bistability governed by cell-to-cell variation in the levels of the sigma factor TcdR, which is directly required for transcription of the toxin genes. TcdR levels are in turn controlled by several metabolic and genetic inputs.

Keywords

sigma factor; signal transduction; toxin regulation; PaLoc; anaerobic RFP; bistability; phase variation

INTRODUCTION

Clostridioides (formerly *Clostridium*) *difficile* is a Gram-positive, spore-forming, anaerobic bacterium and an opportunistic pathogen (Lawson *et al.*, 2016). *C. difficile* infections can cause antibiotic-associated diarrhea and progress to life-threatening conditions, including pseudomembranous colitis and toxic megacolon. Disease is mediated primarily through two exotoxins known as TcdA and TcdB (Lyerly *et al.*, 1982; Lyerly *et al.*, 1985; Triadafilopoulos *et al.*, 1987; Voth and Ballard, 2005). Both toxins are glucosyltransferases that glucosylate host proteins, particularly the Rho family of GTPases (Schirmer and Aktories, 2004; Gerhard *et al.*, 2008; Darkoh *et al.*, 2011; Carter *et al.*, 2012; Shen, 2012; Chandrasekaran and Lacy, 2017). This leads to collapse of the actin cytoskeleton and loss of tight junctions resulting in gastrointestinal distress (Moore *et al.*, 1990; Stubbe *et al.*, 2000; Feltis *et al.*, 2000; Nusrat *et al.*, 2001; Gerhard *et al.*, 2008; Shen, 2012).

TcdA and TcdB are encoded on a 19.6 kb pathogenicity locus (PaLoc), along with three additional toxin-related genes: *tcdR*, *tcdC*, and *tcdE* (Fig. 1) (Cohen *et al.*, 2000). TcdR (σ^{TcdR}) is an alternative sigma factor that recruits RNA polymerase to the promoters for *tcdA* and *tcdB*, and is required for *tcdA* and *tcdB* expression (Mani and Dupuy, 2001). There is also a σ^{TcdR}-dependent promoter upstream of *tcdR*, resulting in a positive-feedback loop whereby σ^{TcdR} increases its own production (Mani *et al.*, 2002). TcdC is proposed to function as an anti-sigma for σ^{TcdR} and thus negatively regulates toxin production (Matamouros *et al.*, 2007; Dupuy *et al.*, 2008; Carter *et al.*, 2011), but this finding has been challenged (Murray *et al.*, 2009; Cartman *et al.*, 2012). TcdE is a predicted membrane protein with some similarity to the holins that create pores for bacteriophage to escape the cytoplasm (Govind and Dupuy, 2012; Govind *et al.*, 2015). Whereas two studies found TcdE is required for toxin secretion (Kai Soo Tan *et al.*, 2001; Govind and Dupuy, 2012), a third study found TcdE to be completely dispensable (Olling *et al.*, 2012).

Regulation of toxin gene expression is one of the best-studied aspects of *C. difficile* biology. When *C. difficile* is cultured in a laboratory in rich media, toxin is produced upon entry into stationary phase (Hundsberger *et al.*, 1997), suggesting expression of *tcdA* and *tcdB* responds to nutrient limitation. Consistent with this view, readily metabolized carbon sources like glucose and a variety of amino acids reduce toxin production (Dupuy and Sonenshein, 1998; Karlsson *et al.*, 1999; Karlsson, S., A. Lindberg, L. G. Burman, E. Norin *et al.*, 2000; Karlsson *et al.*, 2003). These effects are thought to be mediated in part by the

alternative sigma factor σ^{TcdR}, since expression of *tcdR* is influenced by nutrient availability and temperature (Karlsson *et al.*, 2003).

Toxin gene expression in *C. difficile* is also influenced by several global regulators (summarized in Fig. 1 and reviewed in (Bouillaut *et al.*, 2015)). CodY is widely distributed in Firmicutes and functions as a repressor when both GTP and branched chain amino acids are abundant in the cell (Guédon *et al.*, 2001; Ratnayake-Lecamwasam *et al.*, 2001). In *C. difficile*, CodY represses 146 genes, including all five genes in the PaLoc (Dineen *et al.*, 2007; Dineen *et al.*, 2010). *In vitro,* CodY binds to the promoters for *tcdA*, *tcdB*, *tcdC*, and *tcdR*, but because its affinity is about 10-fold higher for the *tcdR* promoter, this is likely to be the most important target for CodY regulation of toxin production (Dineen *et al.*, 2007). CcpA, or carbon catabolite protein A, is a global regulator that responds to readily catabolizable carbohydrates like glucose (Deutscher, 2008). In *C. difficile*, CcpA regulates ~140 genes (Antunes *et al.*, 2012) and binds directly to the promoter regions of *tcdA*, *tcdB*, *tcdC*, and *tcdR* (Antunes *et al.*, 2011). Because CcpA has ~10-fold higher affinity for the *tcdR* promoter than for the other promoters, it is thought to work primarily through controlling expression of *tcdR* (Antunes *et al.*, 2011). Another important regulator is σ^{D}, which is encoded by *sigD* as part of the *flgB* operon that contains early stage flagellar genes (Aubry *et al.*, 2012; El Meouche *et al.*, 2013; McKee *et al.*, 2013). σ^{D} positively regulates *tcdA* and *tcdB* expression by increasing expression of *tcdR* (McKee *et al.*, 2013). A number of other transcriptional regulators have been reported to impact toxin gene expression: PrdR (Bouillaut *et al.*, 2013), Agr (Martin *et al.*, 2013), Spo0A (Mackin *et al.*, 2013), SigH (Saujet *et al.*, 2011), and RstA (Edwards *et al.*, 2016). These are less well understood and some seem to be restricted to certain *C. difficile* strains.

Previous studies of toxin production have focused on populations of cells, and thus reflect the “average” behavior of cells in culture. Our development of RFP as a reporter for gene expression in *C. difficile* enables analysis of *tcdA* and *tcdB* expression at the level of individual cells. Here, we used RFP to study toxin regulation in *C. difficile*. Remarkably, we found toxin gene expression is bimodal; in stationary phase, the population bifurcates into a group of cells that is “TcdA-ON” and a group that is “TcdA-OFF.” In the epidemic strain R20291, about 30% of the cells are in the TcdA-ON state, and the mean fluorescence intensity of these cells is about 50-fold higher than the TcdA-OFF cells. Additional experiments indicate expression of *tcdR* is the genetic switch that determines whether a cell produces toxin.

RESULTS

tcdA expression is bimodal in *C. difficile*

Expression of the toxin genes is induced during stationary phase (Ketley *et al.*, 1984; Osgood *et al.*, 1993; Dupuy and Sonenshein, 1998), and several studies have identified global regulators of *tcdA* expression. However, to date these studies have focused on the bulk population of cells rather than individual cells. We sought to visualize toxin gene expression in single cells by introducing a P_{tcdA}::*rfp* reporter plasmid into R20291 ribotype 027. As expected, in log phase overall fluorescence of the culture as measured with a plate reader was low and fluorescence microscopy revealed the vast majority of the cells were

dark (e.g., sample #1 in Figure 2A, B, C, D). Upon entry into stationary phase overall fluorescence increased ~5 fold and microscopy revealed a striking mixture of bright and dark cells (e.g., sample #5 in Figure 2A, B, C, D). Flow cytometry confirmed that the distribution of fluorescence intensities was bimodal, indicative of two distinct subpopulations, which we will refer to as TcdA-ON and TcdA-OFF. In the experiment shown the TcdA-ON fraction reached a maximum of ~19% of the cells in sample #8 (Fig. 2).

Multiple lines of evidence rule out the potential artifacts of plasmid segregation and viability issues. First, the plasmid is reported to be very stable (Heap *et al.*, 2009; Ransom *et al.*, 2015). Second, an essentially identical plasmid with a lysozyme-induced P_{pdaV}::*rfp* (Ransom *et al.*, 2015) fusion provided uniform red fluorescence across the population after exposure of the cells to lysozyme [See (+), for positive control in Figure 2B, C, D]. As a negative control, a plasmid lacking *rfp* (pRPF185) failed to produce any red fluorescent signal [See (N) in Figure 2B, C, and D]. Third, we sampled cells at various points during the growth curve and plated on TY or TY with thiamphenicol to select for the plasmid. We did not observe a significant drop in cell viability or issues with loss of the reporter plasmid (Fig. S1B–C). Finally, we performed live/dead staining on cells to determine viability. We found that in log phase 100% were viable and after overnight growth 94% remained viable (Fig. S1C–D).

Expression of *tcdA* is bimodal in multiple *C. difficile* ribotypes

As noted, the results shown in Figure 2 were obtained with strain R20291 (ribotype 027), but the *tcdA* promoter region is highly conserved across different *C. difficile* isolates (Fig. S2), suggesting bimodal expression of *tcdA* might be a general property of *C. difficile*. To test this notion, we introduced the P_{tcdA}::*rfp* reporter plasmid into five additional strains: CD630Δerm and JIR8094, which are independent erythromycin-sensitive derivatives of CD630 (ribotype 012); CD196 (ribotype 027); NAP07 (ribotype 078); NAP08 (ribotype 078); and VPI10463 (ribotype 087). The CD196, NAP07, and NAP08 strains are representative clinical isolates corresponding to the most commonly isolated ribotypes of *C. difficile* (Wilcox *et al.*, 2012; Walker *et al.*, 2013), while VPI10463 is commonly known as a high toxin producing strain (Akerlund *et al.*, 2008). As expected, expression of P_{tcdA}::*rfp* was bimodal in all strains, including the three most clinically relevant isolates (Fig. 3). Interestingly, however, two closely related strains, CD630Δerm and JIR8094, exhibited very different fractions of TcdA-ON versus TcdA-OFF subpopulations. In CD630Δerm ~80% of the cells were red fluorescent, as compared to only ~20% in JIR8094 (Fig. 3). As will be explained in more detail below, this difference likely reflects much higher levels of σ^D production in CD630Δerm (Anjuwon-Foster and Tamayo, 2017; Anjuwon-Foster *et al.*, 2018). σ^D increases toxin production by activating *tcdR* transcription (McKee *et al.*, 2013; Anjuwon-Foster and Tamayo, 2017).

Bimodal *tcdA* expression is dependent upon σ^{TcdR}

Bimodal patterns of gene expression can arise from phase variation or from cell-to-cell differences in the levels of a transcriptional activator protein that is part of a positive feedback loop, a mechanism commonly referred to as bistability (Dubnau and Losick, 2006;

Dubnau, 2015). Considering the various factors implicated in control of toxin production in *C. difficile*, the alternative sigma factor σ^{TcdR} is a promising candidate for the control point for bistable toxin gene expression. σ^{TcdR} increases its own expression (Mani *et al.*, 2002), so higher levels of σ^{TcdR} will be self-reinforcing. Moreover, basal expression of *tcdR* is very low (Mani and Dupuy, 2001), creating a situation in which random fluctuations in σ^{TcdR} levels could push a subset of cells over a threshold that locks them into an ON state.

To ask whether *tcdR* might be the genetic switch that determines whether a given *C. difficile* cell is TcdA-ON or TcdA-OFF, we sought to break the positive feedback arising from auto regulation of σ^{TcdR} production. The first step was to construct a *tcdR*::*erm* null mutant in JIR8094 and introduce the P_{tcdA}::*rfp* reporter plasmid. We did not observe any red fluorescence in the *tcdR* null mutant strain (Fig. 4A). The absence of TcdA-ON cells was expected because it is well-established that σ^{TcdR} is essential for transcription of *tcdA* (Mani and Dupuy, 2001; Mani *et al.*, 2002; Karlsson *et al.*, 2003). We then added back a *tcdR* gene under control of a tetracycline-inducible promoter, P_{tet}, which allowed us to control σ^{TcdR} levels by adding increasing amounts of anhydrotetracycline (ATc) to the growth medium. Importantly for our purposes, ATc-induction of P_{tet} is dose-dependent and uniform across a population of cells in *C. difficile* (Fagan and Fairweather, 2011; Ransom *et al.*, 2016). When the *tcdR::erm*/P_{tet}::*tcdR* P_{tcdA}::*rfp* reporter strain was grown in TY broth containing increasing amounts of ATc, we observed a dose-dependent increase in red fluorescence of the cultures (Fig. 4B). Strikingly, however, flow cytometry revealed that red fluorescence was always uniform across the population; there was no concentration of ATc at which *C. difficile* cultures bifurcated into TcdA-ON and TcdA-OFF subpopulations (Fig. 4C). Thus, breaking the positive-feedback loop that controls *tcdR* expression breaks the bimodal expression of *tcdA*, consistent with the hypothesis that toxin gene expression is subject to bistability, and σ^{TcdR} is the master regulator.

σ^{D} influences but is not required for bimodal *tcdA* expression

An alternative underlying cause of bimodal patterns of gene expression is phase variation. In this mechanism, a clonal population of cells becomes genetically heterogenous owing to the generation of (reversible) genetic variants that arise spontaneously. Interestingly, one of the promoters driving production of σ^{TcdR} is recognized by σ^{D}, and σ^{D} expression is subject to phase variation (Anjuwon-Foster and Tamayo, 2017). The gene for σ^{D} is located in the *flgB* operon, which contains flagellar genes and is required for motility (El Meouche *et al.*, 2013; McKee *et al.*, 2013; Anjuwon-Foster and Tamayo, 2017). Expression of the *flgB* operon is regulated by a 154 bp invertible element flanked by 21 bp inverted repeats located between the promoter and the first gene of the operon. It was recently demonstrated that when the invertible element is in the ON orientation σ^{D} is produced, leading to increased expression of *tcdR*, which in turn drives expression of the toxin genes *tcdA* and *tcdB* (Anjuwon-Foster and Tamayo, 2017). Conversely, when the element flips to the OFF orientation, little or no σ^{D} is produced and expression of toxin genes is attenuated (Anjuwon-Foster and Tamayo, 2017).

To explore the role of phase variable production of σ^{D} in generating a bimodal distribution of toxin gene expression, we first asked whether σ^{D} is required for expression of our

P_{tcdA}::*rfp* reporter. To this end, we introduced the reporter plasmid into an R20291 *sigD::erm* mutant and its isogenic wild-type parent (Anjuwon-Foster and Tamayo, 2017). Eliminating σ^D reduced the fraction of red fluorescent (TcdA-ON) cells from ~40% to ~5% of the population, consistent with previous reports that assayed toxin gene expression in a bulk population of cell (Fig. 5A). Nevertheless, for our purposes, the drop in toxin gene expression is less important than the fact that a substantial number of cells express P_{tcdA}::*rfp* despite the complete absence of σ^D. This finding means bimodal *tcdA* expression is not simply a consequence of flagellar inversion at *flgB* and its impact on σ^D levels.

Further evidence that *flgB* phase variation is not required for bifurcation of *C. difficile* into TcdA-ON and TcdA-OFF subpopulations comes from comparison of CD630Δerm and JIR8094. In these strains the invertible element at *flgB* is locked in the ON and OFF orientations, respectively, owing to a mutation in the 21 bp inverted repeats flanking the 154 bp invertible sequence (Anjuwon-Foster *et al.*, 2018). If phase variation at *flgB* were the master regulator of bimodal toxin gene expression, CD630Δerm would be 100% TcdA-ON while JIR8094 would be 100% TcdA-OFF. In reality, however, both strains were bimodal with respect to expression of the P_{tcdA}::*rfp* reporter, with ~80% TcdA-ON in CD630Δerm and ~20% TcdA-ON in JIR8094 (Fig. 3). Our interpretation of these data is that the *flgB* ON orientation biases *C. difficile* towards the TcdA-ON state by driving elevated expression of *sigD*, but other factors impinge upon, and in some cases override, the contribution of phase variation at *flgB*. This renders some cells TcdA-OFF even when σ^D levels are high and some cells TcdA-ON when σ^D is lacking.

We extended these findings by using a P_{tet}::*sigD* construct to assess the effect of modulating *sigD* transcription on expression of the P_{tcdA}::*rfp* reporter. Cultures of a *sigD::erm* mutant harboring a P_{tet}::*sigD*/P_{tcdA}::*rfp* plasmid were grown in TY with increasing amounts of ATc and then assayed for red fluorescence. Although overall fluorescence of the cultures increased with increasing ATc (i.e., increasing σ^D), examination at the level of individual cells revealed bifurcation into TcdA-ON and TcdA-OFF subpopulations across the entire range of inducer concentrations (Fig. 5B). Even at the highest concentration (400 ng/ml) only ~42% of the cells were TcdA-ON (Fig. 5B). Thus, in contrast to a similar experiment performed with P_{tet}::*tcdR*, we were unable to break bimodality by artificially expressing *sigD*. This result provides further evidence that cell-to-cell differences in σ^D abundance are not sufficient to explain the bimodal gene expression of *tcdA*.

The role of environmental signals and global regulators in bimodal *tcdA* expression

Toxin production is influenced by the state of cellular metabolism [reviewed in (Bouillaut *et al.*, 2015)]. For example, exogenous glucose and cysteine reduce toxin production during entry into stationary phase (Karlsson, S., A. Lindberg, L. G. Burman, E. Norin *et al.*, 2000; Antunes *et al.*, 2012). On the other hand, exogenous butyric acid has been reported to increase toxin production (Karlsson *et al.*, 1999; Karlsson, S., A. Lindberg, L. G. Burman, E. Norin *et al.*, 2000). In principle, different levels of toxin production could reflect changes in the fraction of cells that are TcdA-ON, changes in the level of induction of *tcdA* in the TcdA-ON subpopulation, or some combination of the two. We used our P_{tcdA}::*rfp* reporter to examine the effect of glucose, cysteine, and a combination of the two on toxin production

in cells grown in TY. As expected, glucose and cysteine reduced toxin production (Fig. S3). Flow cytometry revealed that glucose and cysteine reduced the fraction of TcdA-ON cells (Fig. S3).

Many global regulators have been reported to influence *tcdR* and thus *tcdA* and *tcdB* gene expression in response to these changes in cellular metabolism [reviewed in (Bouillaut *et al.*, 2015)]. To investigate the role of global regulators on cell-to-cell variation in *tcdA* expression, we introduced the P_{tcdA}::*rfp* reporter plasmid into *C. difficile* JIR8094 TargeTron insertion mutants of four global regulators: *ccpA*, *codY*, *agrB* and *sigH*. Inactivation of *ccpA, agrB,* and *sigH* had almost no effect on bimodal P_{tcdA}::*rfp* expression (Fig. S4). In contrast we observed a 50-fold increase in overall fluorescence in the *codY* null mutant (Fig. S5), which compares favorably with a previous study showing that inactivating *codY* increases *tcdA* mRNA about 50-fold (Dineen *et al.*, 2010). Increased expression of the P_{tcdA}::*rfp* reporter reflected increases in both the number of cells that were TcdA-ON (~3 fold; Fig. 6A and B) and the mean fluorescence intensity of the TcdA-ON cells (~10 fold; Fig. S5). In summary, CodY biases *C. difficile* towards the TcdA-OFF state, but it is not required for bimodality per se as toxin expression remains bimodal in the absence of CodY.

Evidence for bimodal expression of *tcdB*

Because *tcdA* expression is bimodal and σ^{TcdR} levels appear to be critical for establishing bimodality, we hypothesized that expression of toxin B (TcdB) and the master regulator (σ^{TcdR}) would also be bimodal. Unfortunately, wild-type cells carrying a P_{tcdB}::*rfp* reporter plasmid were not fluorescent even though the reporter is on a plasmid present at ~6 copies per cell (Ransom *et al.*, 2015) (Fig. 6C). Apparently, expression of *tcdB* was below our detection limit. The expression of *tcdB* is reported to be 10 to 100 fold lower than *tcdA* (Merrigan *et al.*, 2010; Vohra and Poxton, 2011; Bakker *et al.*, 2012). However, in a *codY*::*erm* mutant background, expression of the P_{tcdB}::*rfp* reporter was readily detected and bimodal (Fig. 6D). We were unable to assess whether production of σ^{TcdR} is bimodal because we could not detect any fluorescence from a P_{tcdR}::*rfp* reporter plasmid, even in a *codY*::*erm* mutant background. Similar reporter constructs incorporating different amounts of DNA from the *tcdR* promoter region were also non-fluorescent. This finding is not too surprising because expression of *tcdR* is known to be lower than that of *tcdA* and *tcdB* (Dupuy and Sonenshein, 1998).

Sporulation and toxin gene expression are not mutually exclusive

The finding that *tcdA* expression is bimodal raises the question: How does differential *tcdA* expression benefit *C. difficile*? It has been proposed that toxin production and sporulation may be mutually (or temporally) exclusive processes in *C. difficile* (Saujet *et al.*, 2011; Bouillaut *et al.*, 2015). Only oxygen-tolerant spores can survive outside the host long enough to be ingested by a new host, but spores are metabolically inert and thus not capable of producing the toxins that cause diarrhea. One clever solution to this conundrum would be for stationary phase cultures of *C. difficile* to differentiate into toxin-producing cells that provoke diarrhea and oxygen-tolerant spores that can survive the journey to the next host. In support of this idea, the master regulator of sporulation, Spo0A, negatively regulates toxin gene expression in some *C. difficile* strain backgrounds (Mackin *et al.*, 2013). In addition,

another positive regulator of sporulation, Spo0H, is said to inhibit toxin production (Saujet *et al.*, 2011). However the relationship between sporulation and toxin production is murky because other studies have come to conflicting conclusions (reviewed in (Martin-Verstraete *et al.*, 2016)).

We used our P_{tcdA}::*rfp* reporter plasmid in conjunction with microscopy to ask whether toxin production and sporulation can occur in the same cell. Cultures of R20291/P_{tcdA}::*rfp* and 630Δerm/P_{tcdA}::*rfp* were sporulated on TY agar containing thiamphenicol to match conditions of our toxin studies. Plates were incubated for 44–76 hrs before samples were harvested and fixed as described previously (Ransom *et al.*, 2016). We were able to identify toxin-producing cells by their red fluorescence and sporulating cells by the fact that spores are phase-bright. Interestingly, TcdA-ON (red) mother cells with spores were readily observed in both strain backgrounds (Fig. 7), and the percentage of cells that were TcdA-ON was similar for vegetative cells and mother cells containing obvious forespores or spores (Table 1). For instance, in R20291, ~15% of cells lacking a spore and ~23% of cells containing a spore were red fluorescent (Table 1). In 630Δerm, which has a much higher percentage of TcdA-ON cells because *flgB* is locked in the ON orientation (Fig. 3; (Anjuwon-Foster *et al.*, 2018)), the corresponding numbers were 83% and 84% TcdA-ON for cells lacking or containing spores, respectively (Table 1). We also observed a small number of free spores that were red fluorescent. These results demonstrate that sporulation and toxin gene expression can occur within the same cell, although they do not rule out the possibility that these events are sequential, i.e., the red fluorescence observed in mother cells containing spores might be residual RFP protein produced prior to entry into the spore developmental program.

DISCUSSION

Toxin gene expression is bimodal in *C. difficile*

C. difficile pathogenesis is mediated primarily by two large exotoxins encoded in the PaLoc, TcdA and TcdB. There has been a lot of effort expended to understand how production of these toxins is controlled. Early studies found that the toxins are produced upon entry into stationary phase (Moncrief and Barroso, 1997; Dupuy and Sonenshein, 1998). This response is mediated by a dedicated sigma factor (σ^{TcdR}) and by a host of global regulatory proteins, most of which sense various aspects of metabolism [reviewed in (Voth and Ballard, 2005; Bouillaut *et al.*, 2015; Martin-Verstraete *et al.*, 2016)]. All these studies have relied on methods that reflect the average behavior of the cells in the population under the (unstated) assumption that toxin production is relatively uniform across the population. Here we have used a fluorescent protein reporter, RFP, to visualize expression of *tcdA* in individual cells. Our results indicate that during entry into stationary phase only a subset of *C. difficile* cells expresses the toxin genes. Expression of the second toxin gene, *tcdB*, was also bimodal, but visualizing this required working in a *codY* mutant background to elevate expression sufficiently to detect it using a fluorescent protein reporter.

Bimodal expression of *tcdA* is probably an example of bistability rather than phase variation, and σ^{TcdR} is the genetic switch

Bimodal distributions of gene expression can arise from phase variation or bistability. Our findings point towards the latter, with the toxin-specific sigma factor σ^{TcdR} being the master regulator that governs the decision between toxin-ON and toxin-OFF. Studies of bistability in other bacteria have revealed two characteristics that make a regulatory protein well-suited for controlling a bistable switch (Dubnau and Losick, 2006). One is low-level basal expression so that stochastic variation can lead to excursions that tip the balance between an ON and an OFF state. The other is a positive feedback loop that reinforces transient increases in cellular abundance of the activator. In the case of toxin gene regulation in *C. difficile*, σ^{TcdR} fulfills both criteria (Dupuy and Sonenshein, 1998; Mani and Dupuy, 2001; Mani *et al.*, 2002). In support of this notion, we found that graded expression of *tcdR* using a tetracycline-inducible promoter prevents development of bistability. Instead, as more inducer is added to the culture, toxin production increases uniformly across the cells in the population. These findings imply that in a wild-type background *tcdR* expression is itself bistable. Unfortunately, efforts to test this idea using an RFP reporter were not successful, owing to the very low level of *tcdR* expression that resulted in levels of RFP below our detection limit.

Multiple global regulators bias cells towards the toxin-ON or toxin-OFF states

A plethora of global regulators have been implicated in control of toxin production in *C. difficile* (Dupuy and Sonenshein, 1998; Dineen *et al.*, 2007; Dineen *et al.*, 2010; Antunes *et al.*, 2011; Saujet *et al.*, 2011; Mackin *et al.*, 2013; El Meouche *et al.*, 2013; McKee *et al.*, 2013). We sought to determine which of these global regulators impacted bistable expression of *tcdA*. Of the major regulators tested, CodY and σ^{D} had the most significant impact on the fraction of TcdA-ON cells; however, neither is required for bistability. In the absence of CodY the fraction of cells expressing *tcdA* increased ~3 fold while the level of toxin production in those cells increased more dramatically by ~10 fold. Nevertheless, populations of the *codY* mutant still bifurcated into TcdA-ON and TcdA-OFF subpopulations, indicating CodY is not responsible for bistability of toxin gene expression per se. *A priori*, the motility sigma factor σ^{D} was a prime candidate for controlling the decision between TcdA-ON and TcdA-OFF because expression of *sigD* is regulated by phase variation (Anjuwon-Foster and Tamayo, 2017). Nevertheless, neither a *sigD::erm* null mutation nor eliminating phase-variable expression of σ^{D} broke bistable expression of the P_{tcdA}::*rfp* reporter. Collectively, our findings indicate σ^{D} biases *C. difficile* towards TcdA-ON, while CodY biases towards TcdA-OFF, but σ^{TcdR} is the master regulator that governs the choice between TcdA-ON and TcdA-OFF.

More generally, *tcdR* expression is affected by multiple physiological inputs and regulatory proteins (Bouillaut *et al.*, 2015; Martin-Verstraete *et al.*, 2016). The sum of these positive and negative inputs poises basal expression of *tcdR* at a given level, be that relatively high or relatively low, which in turn affects the probability that random fluctuations in σ^{TcdR} synthesis and turnover will push cells across a threshold that locks them into the TcdR-ON state that leads to toxin production. For example, when the invertible element at *flgB* is in the ON orientation, expression of *sigD* poises σ^{TcdR} relatively close to the tipping point and

conversion to TcdA-ON is more common. Conversely, in rapidly growing cells replete with energy and amino acids, repression by CodY acting at multiple promoters in the pathogenicity locus renders conversion to TcdA-ON a very rare event.

What are the potential benefits of bistable toxin gene expression?

Bistability has been described as a bet-hedging strategy for dealing with an uncertain and perilous future (Dubnau and Losick, 2006; Davidson and Surette, 2008; Tiwari *et al.*, 2011; García-Pastor *et al.*, 2018). From the perspective of the bacterium, toxin production can be viewed as a means for obtaining food or escaping to a better host. In either case, conditions in the host might change suddenly. If they improve, the large investment in producing the toxins, which are large proteins of over 3000 amino acids that must somehow be transported out of the cell, would be a waste of resources. Conversely, delaying toxin production when conditions are deteriorating comes with its own set of risks. A related possibility is that some of the toxin might remain associated with the cell surface, rendering toxin-producing cells targets for the host immune response. Dilemmas such as these could select for regulatory circuits that incorporate an element of chance into the decision to produce toxins.

An alternative rationale behind bistability invokes division of labor between different cell types needed to achieve a common goal. For example, as noted above, bistable toxin production might be part of a strategy for transmission to a new host whereby some cells produce toxin to provoke diarrhea, while others differentiate into oxygen-tolerant spores that can persist in the environment (Saujet *et al.*, 2011; Bouillaut *et al.*, 2015). However, this explanation seems unlikely as we found that toxin production and spore formation can occur within the same cell.

A third potential explanation for why only a subset of *C. difficile* cells produce toxins is related to how the toxins are released from the cell. The mechanism of toxin release is not yet known. Some studies have implicated a holin-like protein named TcdE in this process, but that finding has been disputed (Govind and Dupuy, 2012; Govind *et al.*, 2015). In the absence of an obvious export apparatus, it has been suggested toxins might be released by cell lysis, with bystanders reaping the benefits. Obviously not all of the cells in a population can afford to lyse. Our data do not support the notion that toxins are released by lysis because we did not observe a large decrease in cell viability when toxin gene expression is artificially induced by either exogenous expression of *tcdR* or deletion of *codY*. Nor did we observe massive lysis of strain CD630Δerm even though 80% of those cells were TcdA-ON. Nevertheless, it remains possible that these mechanisms of driving most cells into the TcdA-ON state do not activate the (putative) lysis mechanisms that might be involved in toxin release. Related to this idea is the potential for toxins to be released when mother cells lyse during spore development. However, this hypothesis implies toxin production would be a step on the pathway towards spore formation, which is inconsistent with our observation that spore development and toxin gene expression appear to be independent phenomena.

Open questions

Using a fluorescent reporter to study toxin gene expression at the level of individual *C. difficile* cells revealed a bistable switch governed by the toxin-specific sigma factor, σ^{TcdR}.

This finding makes it a high priority to better understand how σ^{TcdR} levels are determined. Another key question that remains to be answered is: What benefit does *C. difficile* derive from having only a subset of cells produce toxins? Finally, it bears emphasis that all of the studies presented here were performed in laboratory media. In view of the fact that toxin production responds to metabolic inputs and that growth conditions in the host are very different from those in the lab, it will be interesting to visualize toxin-producing cells in the context of an infection model. Among the important unknowns are what fraction of *C. difficile* cells produce toxins in a host and whether toxin production is restricted to specific regions of the intestines.

MATERIALS AND METHODS

Strains, media, and growth conditions

Bacterial strains used in this study are listed in Table 2. This study included six wild-type *C. difficile* strains: JIR8094, 630Δerm, R20291, CD196, NAP07, and NAP08. *C. difficile* mutants were derived from the erythromycin-sensitive isolate JIR8094, a derivative of the sequenced strain CD630 (Sebaihia *et al.*, 2006; O'Connor *et al.*, 2006). *C. difficile* was routinely grown in Tryptone Yeast (TY) media, supplemented as needed with thiamphenicol at 10 μg/ml, erythromycin at 5 μg/ml, kanamycin at 50 μg/ml, or cefoxitin at 16 μg/ml. TY consisted of 3% tryptone, 2% yeast extract, and 2% agar (for solid media). TY included 0.1% L-cysteine during routine maintenance of *C. difficile* cultures, but cysteine was generally omitted when assaying toxin production. *C. difficile* strains were maintained at 37°C in an anaerobic chamber (Coy Laboratory products) in an atmosphere of 10% H_2, 5% CO_2, and 85% N_2.

Escherichia coli strains were grown in LB medium at 37°C with chloramphenicol at 20 μg/ml and ampicillin at 100 μg/ml as needed. LB contained 1% tryptone, 0.5% yeast extract, 1% NaCl and, for plates, 1.5% agar.

Plasmid and strain construction

All plasmids are listed in Table 3. Regions of plasmids constructed using PCR were verified by DNA sequencing. The oligonucleotide primers used in this work were synthesized by Integrated DNA Technologies (Coralville, IA). Primers are listed in Table S1. All plasmids were constructed using OmniMax-2 $T1^R$ as the cloning host, transformed into HB101/pRK24, and then introduced into *C. difficile* strains by conjugation (Trieu-Cuot *et al.*, 1987).

The *C. difficile* null mutant of $tcdR_{142}$ was constructed using modified TargeTron procedures (Sigma-Aldrich) to insert a group II intron conferring Erm resistance (Heap *et al.*, 2007; Heap *et al.*, 2010; Ho and Ellermeier, 2011). Primers for retargeting the group II intron were designed using the ClosTron algorithm (Heap *et al.*, 2010). To retarget the intron to insert after nucleotide 142 of *tcdR*, the intron template was amplified by PCR as outlined in the TargeTron user manual (Sigma-Aldrich) using an EBS universal primer designated CDE914 in combination with primers RP398, RP399, and RP400. The resulting PCR product and the vector pBL100 (Bouillaut *et al.*, 2013) were digested with HindIII and BsrGI, and then ligated to create plasmid pRAN1034. pRAN1034 was transferred to *C.*

difficile JIR8094 via conjugation and isolates in which the intron had moved to the $tcdR_{142}$ locus were obtained by selection for Erm-resistance as described previously (Heap *et al.*, 2010; Ho and Ellermeier, 2011). Intron insertion into $tcdR_{142}$ was confirmed by PCR. Finally, loss of the TargeTron plasmid was confirmed by thiamphenicol sensitivity. To construct additional TargeTron mutants the following primer combinations were used to generate vectors for mutagenesis: $codY_{330}$, RP323-RP325, pCE536; $ccpA_{133}$, RP326-RP328, pCE541; $agrB_{188}$ CDEP1807-CDEP1809, pTHE538; and $sigH_{123}$, RP335-RP337, pCE540. Mutagenesis was carried out as described above.

For expression studies plasmids were constructed with promoters from *tcdA*, *tcdB*, and *tcdR*. The plasmids are all derivatives of pDSW1728, which has a tetracycline-inducible promoter and codon optimized Red Fluorescent Protein mCherryOpt (P_{tet}::*rfp*) (Ransom *et al.*, 2015; Ransom *et al.*, 2016). Promoters were amplified using the following primer sets: P_{tcdA} (RP304 and RP305), P_{tcdB} (RP345 and RP346), and P_{tcdR} (RP347 and RP348). The PCR products were digested with NheI and SacI, then ligated into pDSW1728 digested with the same enzymes to cut out the P_{tet} promoter. The resulting plasmids were designated pRAN737 (P_{tcdA}), pRAN841 (P_{tcdB}), and pRAN842 (P_{tcdR}).

To regulate *tcdR* expression in *C. difficile*, we built two constructs that had *tcdR* under an inducible promoter: P_{tet} (Fagan and Fairweather, 2011) or P_{pdaV} (Ho and Ellermeier, 2011; Ho *et al.*, 2014; Ransom *et al.*, 2015). To build P_{pdaV}::*tcdR*, a synthetic DNA fragment (gBlock) containing both the promoter and gene was synthesized by Integrated DNA Technologies, and the fragment was amplified using RP374 and RP375. This DNA was digested with XmaI and inserted into the XmaI site of pRAN737. The resulting plasmid was designated pRAN1018 (P_{tcdA}::*rfp*; P_{pdaV}::*tcdR*). To build P_{tet}::*tcdR*, the P_{tet} promoter was amplified by PCR using primers RP393 and RP394, with pRPF185 as the template (Fagan and Fairweather, 2011). The P_{tet} promoter was then swapped with the P_{pdaV} promoter in pRAN1018 using a KpnI and SphI digest and ligation. The resulting plasmid was named pRAN1032 (P_{tcdA}::*rfp;* P_{tet}::*tcdR*).

To build P_{tet}::*sigD*, the *sigD* gene was amplified from R20291 chromosomal DNA using primers CDEP3531 and CDEP3532. pRAN1032 was digested using SphI and AscI to remove *tcdR*. The *sigD* PCR product was inserted into the cut vector using isothermal assembly resulting in plasmid pGK110.

Fixation protocol

Cells were fixed as previously described (Ransom *et al.*, 2014; Ransom *et al.*, 2016). Briefly, a 500-μl aliquot of cells in growth medium was added directly to a microcentrifuge tube containing 120 μl of a 5X fixation cocktail: 100 μl of 16% (wt/vol) paraformaldehyde aqueous solution (methanol-free; catalog no. AA433689M; Alfa Aesar, Ward Hill, MA) and 20 μl of 1 M $NaPO_4$ buffer (pH 7.4). The sample was mixed, allowed to sit for 15 min, removed from the Coy chamber, and incubated on ice for 45 min. The fixed cells were washed three times with PBS, resuspended in 30 μl of PBS, and left in the dark at 4°C to allow for chromophore maturation.

Microscopy

Microscopy was performed as described previously (Ransom *et al.*, 2016). Cells were immobilized using thin agarose pads (1%). Phase-contrast and fluorescence micrographs were recorded on an Olympus BX60 microscope equipped with a ×100 UPlanApo objective (numerical aperture, 1.35). For RFP the filter set (catalog no. 41004) comprised a 538- to 582-nm excitation filter, a 595-nm dichroic mirror (long pass), and a 582- to 682-nm emission filter. This filter set was from Chroma Technology Corp. (Brattleboro, VT). Micrographs were captured with a Spot 2 CCD camera as described (Ransom *et al.*, 2014; Ransom *et al.*, 2015; Ransom *et al.*, 2016) or with a Hamamatsu ORCA Flash 4.0 V2+ CMOS camera. Typical exposure times for RFP were 3 seconds for the Spot camera and 250 milliseconds for the Flash 4.0 camera. To ensure comparability of fluorescence micrographs, the display range option was adjusted identically for all images. Micrographs were cropped, and figures were assembled in Adobe Illustrator (Adobe Systems, Inc., San Jose, CA) or Olympus cellSens Dimension software.

Flow cytometry

Cells were analyzed at the Flow Cytometry Facility at the University of Iowa. The equipment used in this study includes the Becton Dickinson LSR II with a 561nm laser, 610/20 bandpass filter, and 600 LP dichroic filter, and the Becton Dickinson Aria II. Data was analyzed using BD FACSDiva Software.

Fluorescence measurements with a plate reader

The plate reader was used to measure bulk samples from cultures as described previously (Ransom *et al.*, 2016). Briefly, fluorescence and absorbance (OD_{600}) were measured with an Infinite M200 Pro plate reader (Tecan, Research Triangle Park, NC). Samples were prepared by adding 20 µl of fixed cells in PBS and 180 µl of PBS to the well of a flat-bottom 96-well microtiter plate (AS Plate-PS-96-F-C; AG Advangene, IL). Fluorescence was recorded as follows: excitation, 554 nm; emission, 610 nm; gain setting, 100. The cell density (OD_{600}) was also recorded and used to normalize the fluorescence reading.

Spore Preparation

C. difficile spores were obtained following standard procedures as previously described (Edwards and McBride, 2016). Briefly, *C. difficile* strains were grown overnight in TY broth with 10 µg/ml thiamphenicol. 200 µl of overnight culture was plated on TY agar with 10 µg/ml thiamphenicol. Plates were incubated at 37°C for 44–76 hours. Cells were scraped from plates and suspended in 500 µl TY broth. Samples were then fixed and visualized as described above.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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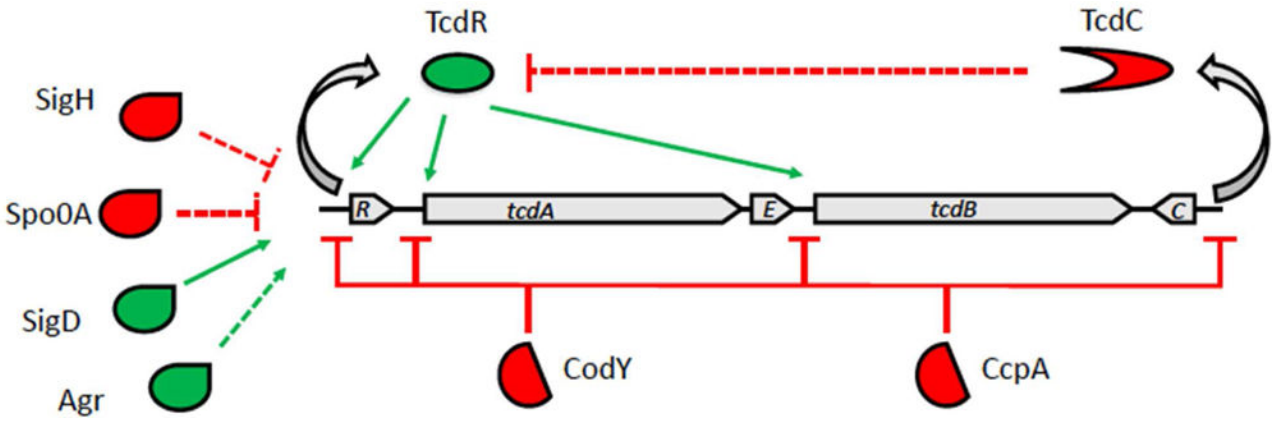

Figure 1. Model for regulation of toxin gene expression.

Positive and negative regulators are shown in green and red, respectively. Dashed lines indicate factors that may act indirectly and/or whose contributions are not clearly established. Note that σ^{TcdR} activates expression of its own gene (*tcdR*) and both toxin genes *tcdA* and *tcdB*, while σ^{D} activates expression of *tcdR*. The repressor CodY binds to the promoter-regulatory regions at *tcdR*, *tcdA*, and *tcdB*.

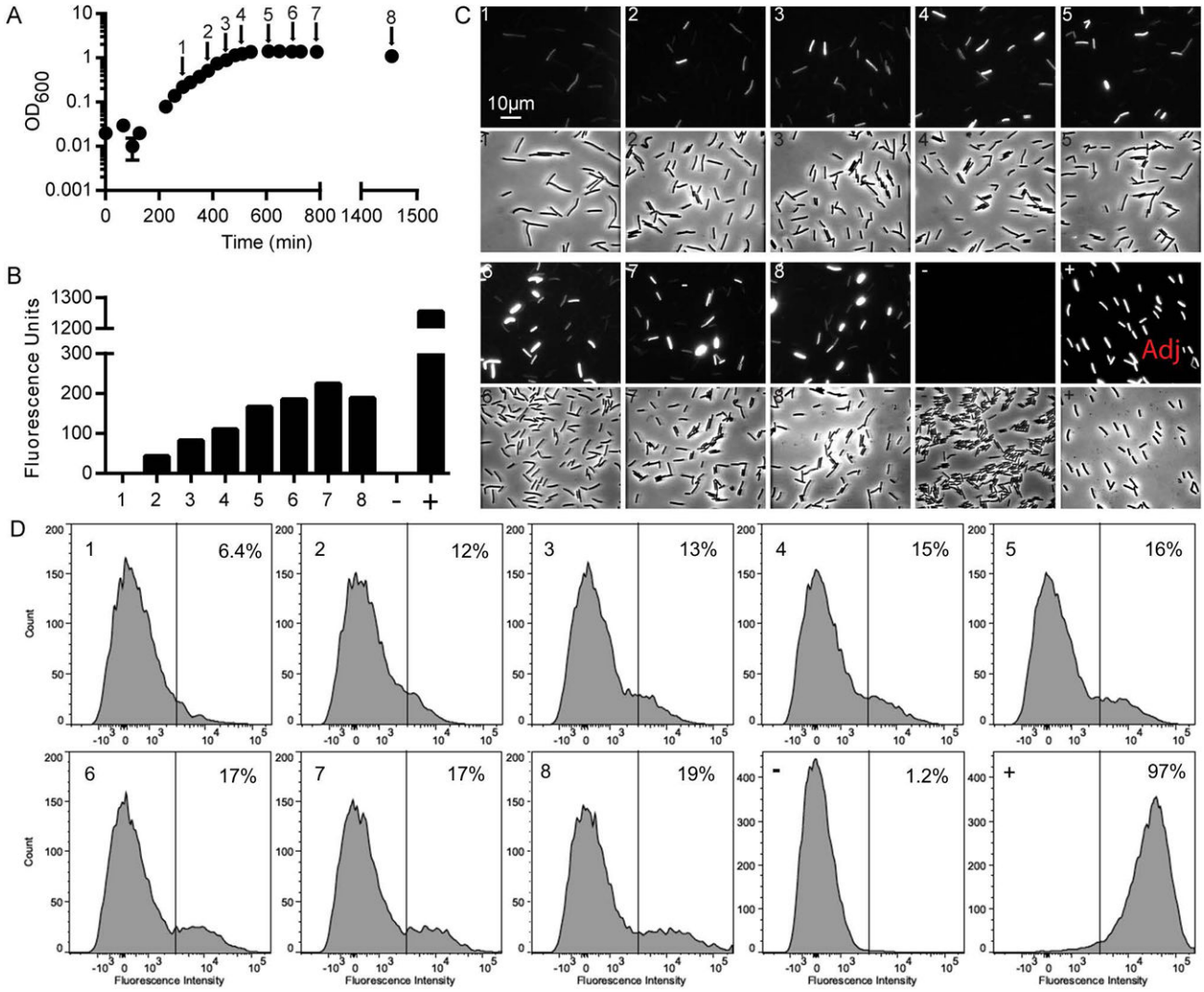

Figure 2. Bistable expression of P_{tcdA}::*rfp* in *C. difficile* R20291.

(A) Growth curve of P_{tcdA}::*rfp* strain. At the times indicated by the arrows, samples of the culture were fixed with paraformaldehyde and exposed to air to allow red fluorescence to develop. **(B)** Specific fluorescence of P_{tcdA}::*rfp* population as determined using a plate reader. Numbers on the x-axis refer to samples fixed at the time points indicated in (A). Also shown is a negative control strain containing a P_{tet}::*gus* reporter plasmid (−) and a positive control strain carrying a P_{pdaV}::*rfp* reporter plasmid and induced with lysozyme for 30 min (+). **(C)** Expression of P_{tcdA}::*rfp* as assessed by microscopy. Micrographs are paired: fluorescence (above) and phase micrographs (below). All micrographs were captured and processed identically except for one marked “Adj” which indicates the brightness was adjusted down. **(D)** Expression of P_{tcdA}::*rfp* as assessed by flow cytometry. The x-axis label is Fluorescence Intensity in arbitrary units. Vertical gate divides TcdA-ON from TcdA-OFF subpopulations, and numbers in the upper right corner of each panel refers to the fraction of cells scored as TcdA-ON.

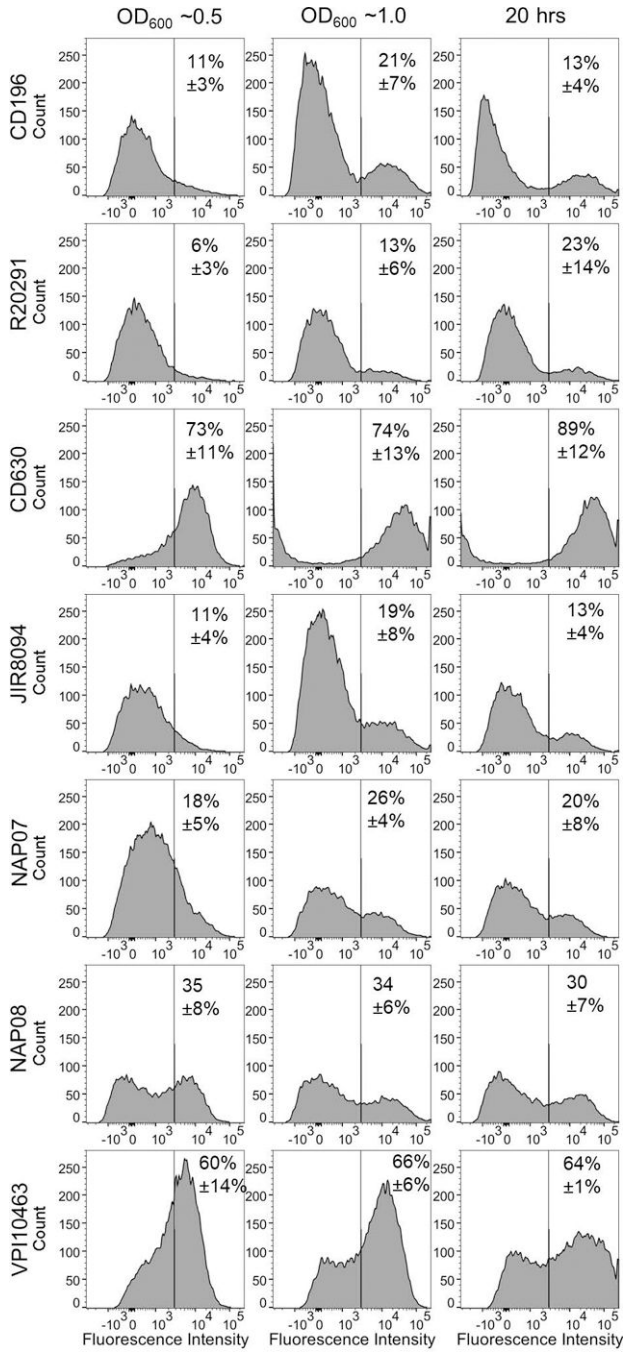

Figure 3. Expression of P_{tcdA}::*rfp* is bistable in multiple *C. difficile* strains.

The indicated strains of *C. difficile* harboring the P_{tcdA}::*rfp* reporter plasmid were fixed at **(A)** OD_{600} ~0.5, **(B)** OD_{600} ~1.0 and **(C)** after 20 hours of growth. Percentages refer to the fraction of cells that were TcdA-ON (mean ± st. dev., n = at least 3 independent experiments). The strains shown represent the following ribotypes: CD196 (ribotype 027), R20291 (ribotype 027), CD630Δerm (ribotype 012), JIR8094 (ribotype 012), NAP07 (ribotype 078), NAP08 (ribotype 078) and VPI10463 (ribotype 087).

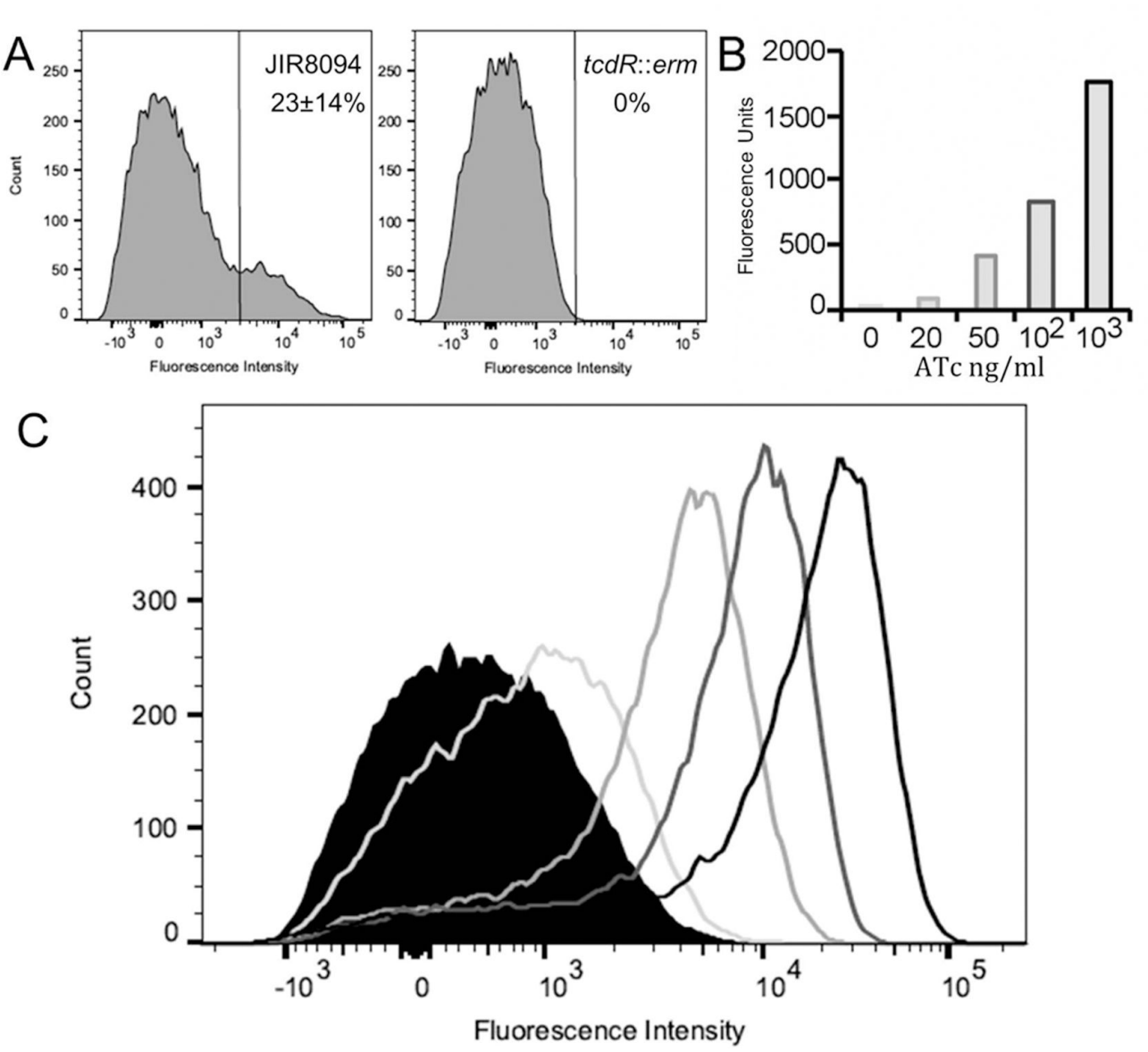

Figure 4. TcdR mediates bistable expression of *tcdA*.

(A) TcdR is required for *tcdA* expression. Wild-type JIR8094/P$_{tcdA}$::*rfp* and *tcdR*::*erm*/P$_{tcdA}$::*rfp* were grown to stationary phase (24 hrs). Samples of each culture were fixed and analyzed for red fluorescence by flow cytometry. **(B, C)** Breaking the positive feedback loop that controls *tcdR* expression prevents development of bistability. The *tcdR*::*erm* mutant harboring a plasmid with both P$_{tet}$::*tcdR* and P$_{tcdA}$::*rfp* was grown to mid-log phase (OD$_{600}$ = 0.3), at which time ATc was added as indicated to induce expression of *tcdR*. After 1 hr, samples were fixed and analyzed using a plate reader **(B)** or by flow cytometry **(C)**. Data shown are from one experiment that is representative of three trials.

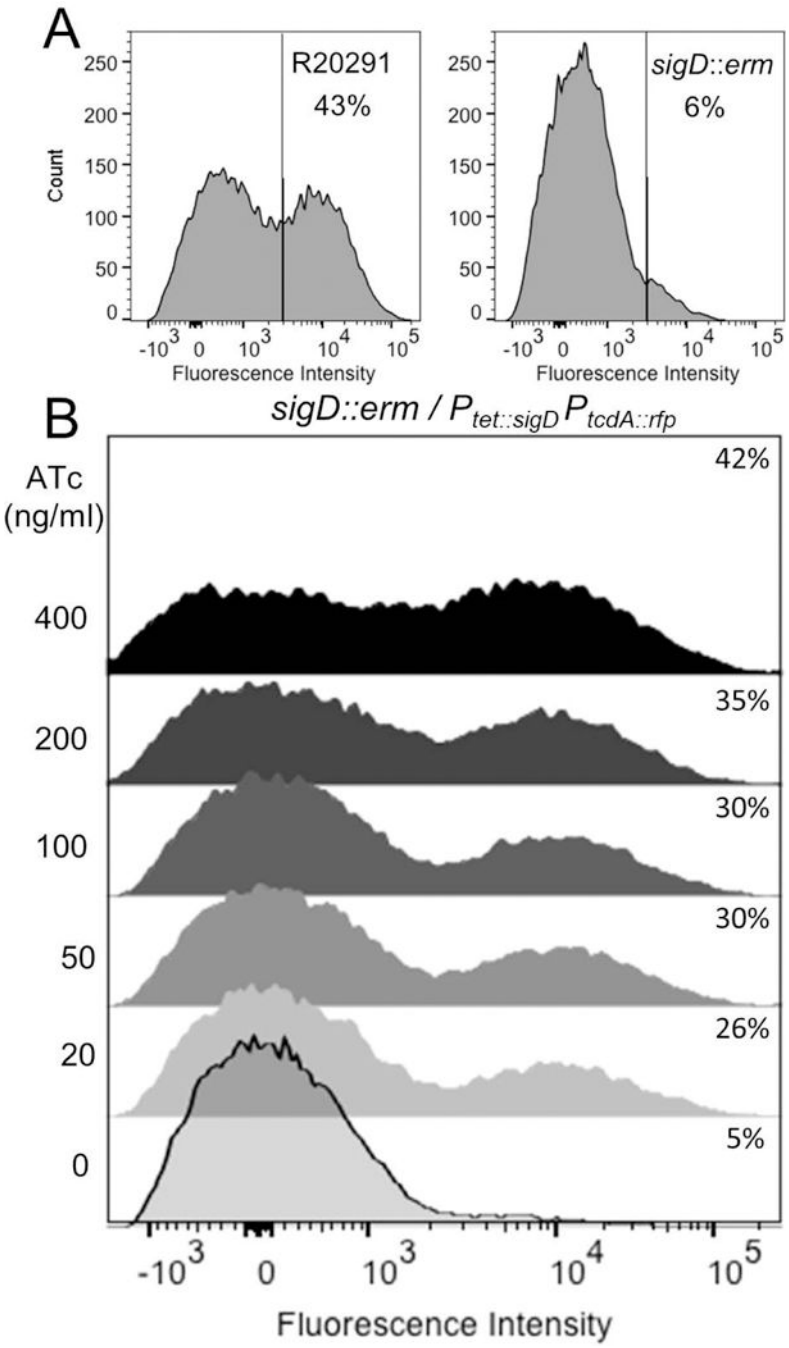

Figure 5. σ^D impacts but is not required for bistable expression of *tcdA*.

(A) σ^D is not essential for *tcdA* expression. Wild-type R20291/P_{tcdA}::*rfp* and *sigD*::*erm*/P_{tcdA}::*rfp* were grown to stationary phase (24 hrs). Samples of each culture were fixed and analyzed for red fluorescence by flow cytometry. **(B)** The *sigD*::*erm* mutant harboring a plasmid with both P_{tet}::*sigD* and P_{tcdA}::*rfp* was grown to mid-log phase (OD_{600} = 0.3), at which time ATc was added as indicated to induce expression of *sigD*. After 2 hrs, samples were fixed and analyzed by flow cytometry. Data shown are from one experiment that is representative of three trials.

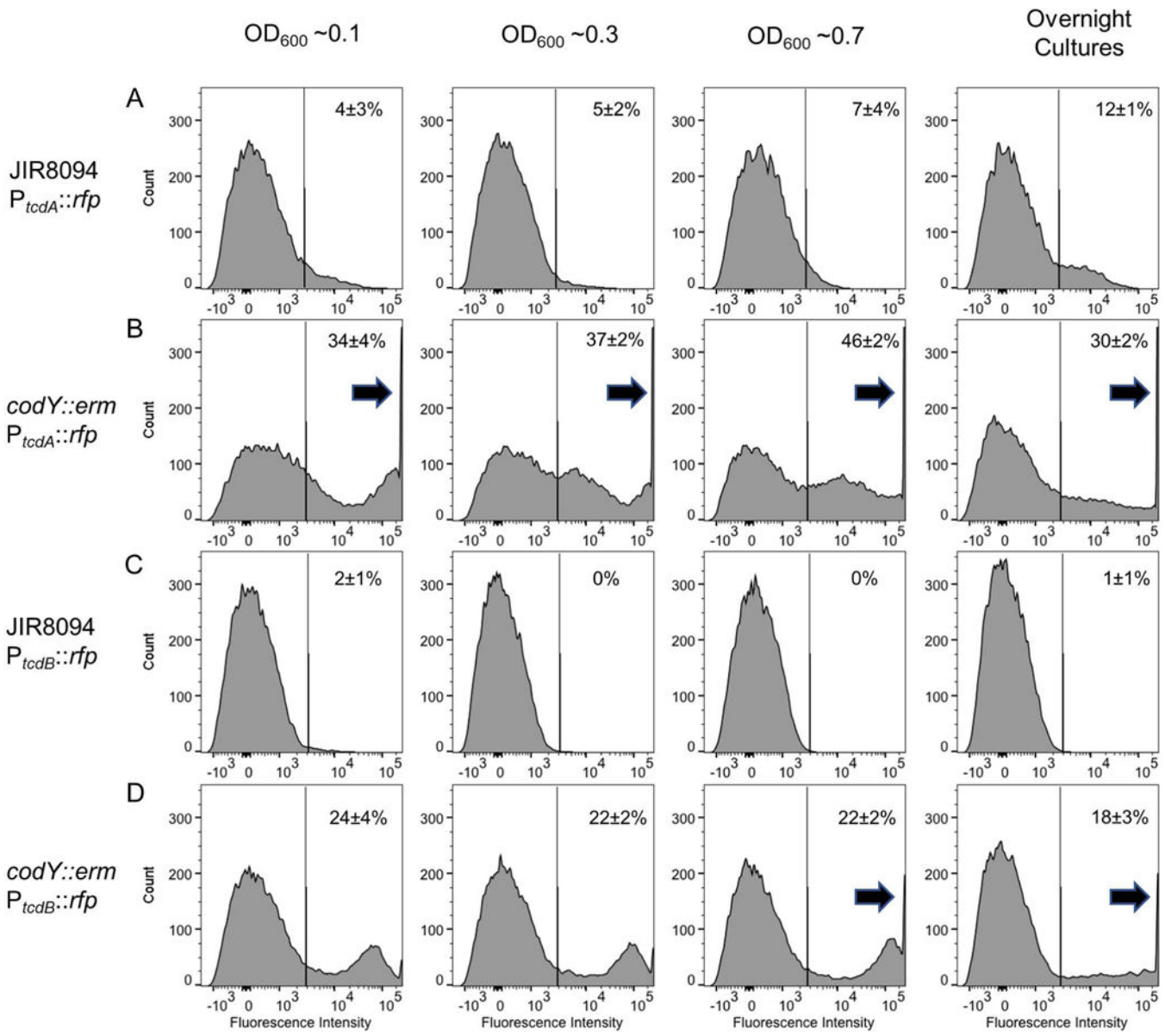

Figure 6. CodY impacts but is not essential for bimodal *tcdA* expression.

The indicated strains wild-type JIR8094/P*tcdA*::*rfp, codY*::*erm*/P*tcdA*::*rfp,* JIR8094/P*tcdA*::*rfp* and *codY*::*erm*/P*tcdA*::*rfp* were grown in TY and samples were harvested at the OD_{600} noted. Samples were fixed and analyzed by flow cytometry. Note the large number of cells in the *codY* mutant strains that saturated the fluorescence detector during flow cytometry (arrows). Percentages refer to fraction of TcdA-ON or TcdB-ON cells (mean ± st. dev., n = 3 experiments).

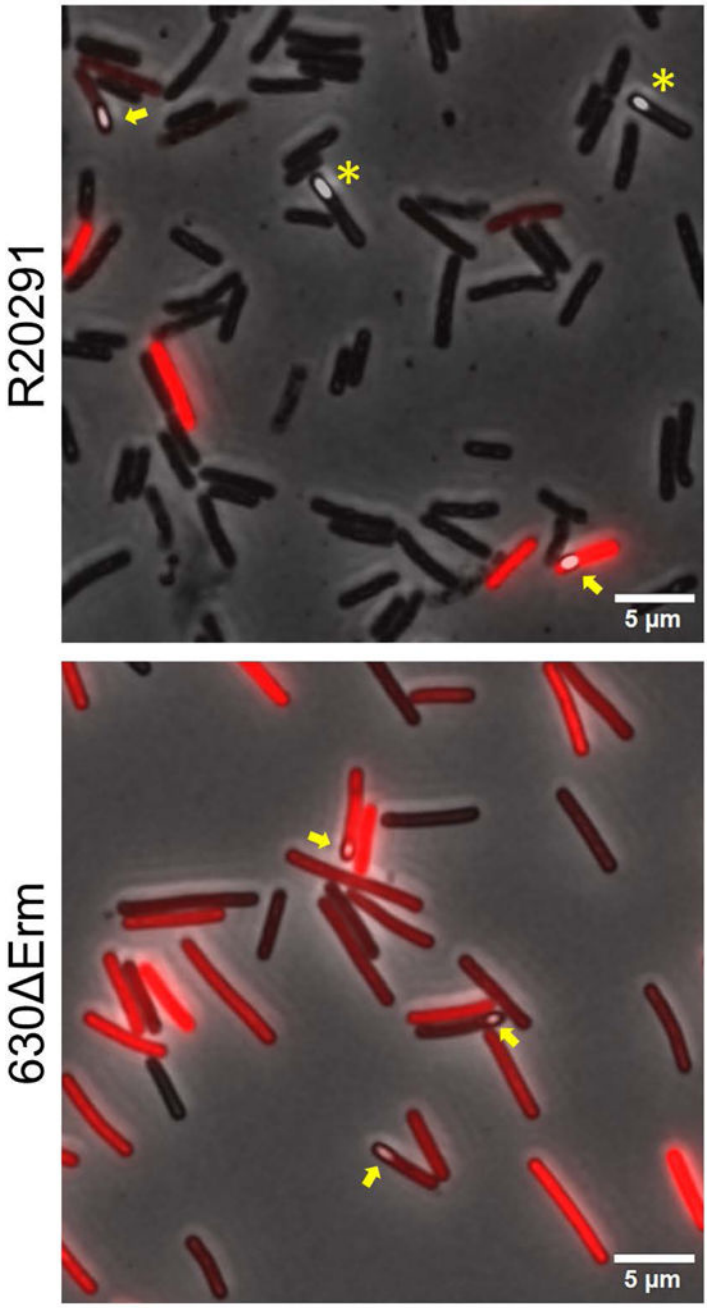

Figure 7. Sporulation and toxin expression are independent processes.

C. difficile strains R20291 and CD630Δerm harboring pRAN737 (P_{tcdA}::*rfp*) were sporulated on TY agar with thiamphenicol. Cells and spores recovered from plates were fixed, removed from the anaerobic chamber to allow RFP to mature, and then imaged by phase contrast and fluorescence microscopy. Shown are overlays of the phase-contrast images and fluorescence images. Yellow arrows indicate examples of toxin-expressing cells that contain a developing spore. Yellow asterisks indicate sporulating cells that are not expressing the *tcdA* reporter.

Table 1.

Spore formation and toxin expression

	% expressing P_{tcdA}-*rfp*	Total cells counted**
R20291		
Vegetative	15.1%	4580
Forespores*	22.5%	129
Endospores*	19.6%	46
630Δerm		
Vegetative	83%	2391
Forespores*	84%	88
Endospores*	33%	3

*Forespores refers to mother cells containing a phase-bright spore while endospores refers to free spores.

**Data pooled from 3 experiments that yielded similar results.

Table 2.

Strains

Strain	Genotype and Description	Reference*
E. coli		
OmniMAX – 2 T1^{R}	F´ [*pro*AB+ *lac*Iq *lac*ZΔM15 Tn10(Tet^{R}) Δ(*ccd*AB)] *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) Φ80*lac*ZΔM15 Δ(*lac*ZYA-*arg*F) U169 *end*A1 *rec*A1 *sup*E44 *thi*-1 *gyr*A96 *rel*A1 *ton*A *pan*D.	Invitrogen
XL1-Blue	*endA1 gyrA96*(nal^{R}) *thi-1 recA1 relA1 lac glnV44* [F' *proAB*$^{+}$ *lacI*^{q} Δ(*lacZ*)M15] *hsdR17*(r_K^- m_K^+) Tn*10*(Tet^{R})]	
HB101/pRK24	F− *mcrB mrr hsdS20*(r_B^- m_B^-) *recA13 leuB6 ara-14 proA2 lacY1 galK2 xyl-5 mtl-1 rpsL20*	(Trieu-Cuot *et al.*, 1987)
EC3272	XL1-Blue / pDSW1728 (P_{tet}::*rfp cat*)	(Ransom *et al.*, 2015)
RAN473	OmniMAX / pRAN473 (P_{tet}::*rfp* –MCS *cat*)	(Ransom *et al.*, 2015)
C. difficile		
JIR8094	Spontaneous erythromycin-sensitive derivative of strain 630 (Ribotype 012)	(O'Connor *et al.*, 2006)
CD630Δerm	Spontaneous erythromycin-sensitive derivative of strain 630 (Ribotype 012)	(Hussain *et al.*, 2005)
CD196	Wild-type *C. difficile* strain from France (ribotype 027)	
NAP07	Wild-type *C. difficile* strain ribotype 078	
NAP08	Wild-type *C. difficile* strain ribotype 078	
R20291	Wild-type *C. difficile* strain from UK outbreak (ribotype 027)	
RAN820	NAP08 / pRAN737 (P_{tcdA}::*rfp cat*)	
RAN828	JIR8094 / pRAN737 (P_{tcdA}::*rfp cat*)	
RAN829	JIR8094 / pRAN737 (P_{pdaV}::*rfp cat*)	(Ransom *et al.*, 2015)
RAN912	JIR8094 / pRAN841 (P_{tcdB}::*rfp* cat)	
RAN913	JIR8094 / pRAN842 (P_{tcdR}::*rfp* cat)	
RAN925	R20291 / pRAN737 (P_{tcdA}::*rfp cat*)	
RAN934	CD196 / pRAN737 (P_{tcdA}::*rfp cat*)	
RAN1101	NAP07 / pRAN737 (P_{tcdA}::*rfp cat*)	
GMK134	CD630Δerm / pRAN737 (P_{tcdA}::*rfp cat*)	
RAN1116	JIR8094 $codY_{330}$::*ltrB*::*ermB*	
RAN1123	JIR8094 $tcdR_{142}$::*ltrB*::*ermB*	
RAN1121	JIR8094 $sigH_{123}$::*ltrB*::*ermB*	
RAN1120	JIR8094 $ccpA_{133}$::*ltrB*::*ermB*	
CDE1774	JIR8094 $agrB_{188}$::*ltrB*::*ermB*	
RAN1124	JIR8094 $codY_{330}$::*ltrB*::*ermB* / pRAN737 (P_{tcdA}::*rfp cat*)	
RAN1127	JIR8094 $tcdR_{142}$::*ltrB*::*ermB* / pRAN737 (P_{tcdA}::*rfp cat*)	
RAN1126	JIR8094 $sigH_{123}$::*ltrB*::*ermB* / pRAN737	
RAN1125	JIR8094 $ccpA_{133}$::*ltrB*::*ermB* / pRAN737	
CDE2770	JIR8094 $agrB_{188}$::*ltrB*::*ermB* / pRAN737	
TCD20	JIR8094 $csfV_{63}$::*ltrB*::*ermB*	(Ho *et al.*, 2014)

Strain	Genotype and Description	Reference*
RAN1129	JIR8094 *tcdR*$_{142}$::*ltrB*::*ermB* / pRAN1032	
RT1566	R20291 *sigD*$_{228}$::*erm*	(Anjuwon-Foster and Tamayo, 2017)
GMK129	R20291 *sigD*$_{228}$::*erm* / pRAN737	
GMK130	R20291 *sigD*$_{228}$::*erm* / pGK110	

* This study unless otherwise noted.

Table 3.

Plasmids

Plasmid	Relevant Features	Reference*
pRPF185	*E. coli-C. difficile* shuttle vector with tetracycline-inducible promoter. P_{tet}::*gusA cat CD6ori RP4oriT-traJ pMB1ori*	(Fagan and Fairweather, 2011)
pBL100	*E. coli-C. difficile* shuttle vector for creating *C. difficile* mutants using TargeTron mutagenesis. *ltrB*::*ermB*::*RAM ltrA cat bla CD6ori RP4oriT pMB1ori*	(Bouillaut *et al.*, 2013)
pDSW1728	P_{tet}::*rfp cat*	(Ransom *et al.*, 2015)
pCE536	pBL100 targeted to $codY_{330}$	
pRAN737	pDSW1728 derivative with P_{tcdA}::*rfp*	
pRAN841	pDSW1728 derivative with P_{tcdB}::*rfp*	
pRAN842	pDSW1728 derivative with P_{tcdR}::*rfp*	
pRAN1018	P_{tcdA}::*rfp* / P_{pdaV}::*tcdR*	
pRAN1032	P_{tcdA}::*rfp* / P_{tet}::*tcdR*	
pTHE583	pBL100 targeted to $agrB_{188}$	
pCE540	pBL100 targeted to $sigH_{123}$	
pCE541	pBL100 targeted to $ccpA_{133}$	
pRAN1034	pBL100 targeted to $tcdR_{142}$	
pGK110	P_{tcdA}::*rfp* / P_{tet}::*sigD*	

*This study unless otherwise noted.