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Regulation of *Clostridioides difficile* toxin production

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Abstract

Clostridioides difficile produces toxins TcdA and TcdB during infection. Since the severity of the illness is directly correlated with the level of toxins produced, researchers have long been interested in the regulation mechanisms of toxin production. The advent of new genetics and mutagenesis technologies in *C. difficile* has allowed a slew of new investigations in the last decade, which considerably improved our understanding of this crucial regulatory network. The current body of work shows that the toxin regulatory network overlaps with the regulatory networks of sporulation, motility, and key metabolic pathways. This implies that toxin production is a complicated process initiated by bacteria in response to numerous host factors during infection. We summarize the existing knowledge about the toxin gene regulatory network here.

Keywords

C. difficile; Clostridioides difficile; toxin gene regulation; virulence gene regulation

Introduction

Clostridioides difficile has increasingly become one of the most clinically relevant human pathogens in the last decade. The frequency and severity of *C. difficile* infection (CDI) has increased worldwide to become one of the most common hospital-acquired infections, especially in industrialized countries [1]. CDI symptoms range from mild diarrhea to severe life-threatening pseudomembranous colitis and toxic megacolon [2]. These symptoms are essentially caused by the production of two major virulent factors, Toxin A (TcdA) and Toxin B (TcdB) [3]. Under laboratory conditions, production of TcdA and TcdB occurs in responce to various environmental conditions, such as availability of specific nutrients, temperature, cell density, redox potential changes, phage infection, and presence of antibiotics [4–11]. This review will provide a comprehensive discussion on the regulatory networks that control the production of TcdA and TcdB.

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Declaration of Interest
None

Conflict of Interest:
None exist

Regulators located in PaLoc (Pathogenicity Locus)

In *C. difficile*, the genes *tcdA* and *tcdB* are located in the pathogenicity locus (Paloc), a 19.5 kb chromosomal region found only in the toxigenic *C. difficile* strains. Besides the toxin genes, PaLoc consists of *tcdR*, *tcdC* and *tcdE*. TcdR and TcdC are involved in transcriptional regulation of the toxin genes [12,13], and TcdE might be needed for the efficient secretion of toxins [14,15]. While the function of TcdR as a positive regulator is well established, the roles of TcdC and TcdE are still under investigation.

TcdR is a group V sigma factor homologous to toxin gene regulators of other clostridial pathogens, such as TetR of *Clostridium tetani* and BotR of *Clostridium botulinum* [reviewed in 16]. TcdR activates transcription by directing the RNAP from its own promoter and the promoters of *tcdA* and *tcdB* [12]. Utilizing single-cell analysis with a red fluorescent protein (RFP) as a reporter, Ransom *et al*. showed that TcdR accumulates to trigger toxin production only in a subset of cells in the population [17]. Thus, TcdR concentration appears to control a bistable switch that turns the toxin genes ON or OFF in a cell.It is important to note that TcdR is the only positive regulator capable of directly controlling the transcription of the toxin genes. Many known positive and negative regulators modulate *tcdR* expression and indirectly influence toxin gene transcription (Figure 1) [18–21].

TcdC has long been thought to negatively affect toxin gene expression due to the inverse timing of its expression compared to the other PaLoc genes [22]. TcdC has been suggested to act as an anti-sigma factor by interfering with the capacity of the RNAP-TcdR complex to recognize the *tcdA* and *tcdB* promoters [13]. Carter *et al*. utilized a plasmid-borne copy of the *tcdC* gene to complement the *C. difficile* NAPI/027 strain (M7404) lacking the functional *tcdC* gene and showed that *tcdC* expression down-regulated toxin production and reduced pathogenicity [23]. Even though these studies suggested *tcdC*'s role in toxin gene regulation, other studies generated contradictory results [24,25]. TcdC, unlike any other known regulatory protein, does not have a DNA-binding domain. It is a membrane-associated protein with a transmembrane domain at the N-terminus [13,26]. Its C-terminal region was thought to be localized in the cytoplasm to interact with TcdR [13]. More recently, the HiBiT extracellular detection system demonstrated that the C- terminus of TcdC is located extracellularly [27]. This calls into question the present model of TcdC's anti-sigma factor mechanism of action. More research is needed to fully understand the role of TcdC in *C. difficile* physiology and toxin gene regulation.

Toxin gene regulation in response to nutrients

Significant variations in the level of toxin production in *C. difficile* strains are seen in the presence of different nutrient sources. Glucose, certain amino acids and butyrate in the medium greatly influence toxin production [6,8,9]. Molecular pathways controlling *C. difficile* toxin gene expression in response to some of these nutrients involve major regulators, CcpA, CodY, PrdR, and Rex [18,19,28–30].

The effects of rapidly metabolizable sugars on toxin gene expression are examples of carbon catabolite repression (CCR). In this phenomenon where the presence of a carbon source in the medium represses the expression of certain genes and operons. CcpA, a Lac repressor

family protein, is the main agent of CCR in low G+C Gram-positive bacteria [18]. CcpA reduces toxin gene expression in *C. difficile* by interacting directly with the PaLoc promoter regions, having the greatest affinity for the *tcdR* promoter region. [18]. In a *ccpA* mutant, toxin production remains the same irrespective of the presence or absence of glucose, however lower than in the parent strain grown without glucose [18]. This finding implies that CcpA also controls other regulators involved in toxin gene regulation. Transcriptomics analysis of the *ccpA* mutant indeed revealed CcpA directly or indirectly controlling several other global regulators, including CodY and Rex [18].

While CcpA controls toxin production in reaction to glucose, repression of toxin synthesis in response to amino acids is mediated by CodY. During the exponential growth phase, when the nutrients are abundant, CodY binds to branched-chain amino acids (BCAAs)/ or GTP and acts primarily as a repressor of various alternative metabolic pathways [30,31]. When nutrients become limited in the cell, CodY is no longer bound by the cofactors, and the transcriptional repression by CodY is alleviated. CodY controls toxin gene expression by binding to the *tcdR* promoter region at three distinct sites with variable affinities, two of which overlap with the TcdR binding sites. In addition to its direct action on the toxin gene locus, CodY also regulates other metabolic pathways that affect toxin production. For example, genes involved in the butyrate synthesis and c-di-GMP metabolism are direct targets of CodY and are repressed when BCAA and GTP are in excess in the medium [31–33]. CodY also regulates the master regulators SinR and Spo0A, which are known to govern toxin production (see below) (Figure 1).

Cysteine is one of the most potent amino acids that down-regulates toxin production in *C. Difficile* [8]. Through an unknown mechanism, SigL, which belongs to the σ54 family, has been identified to promote cysteine-dependent suppression of toxin synthesis, probably in response to the accumulation of by-products of cysteine degradation, such as pyruvate (Figure 1) [34]. Similar to cysteine, the amino acids proline and glycine also have a strong inhibitory effect on the production of toxins [9]. Both proline and glycine are part of Stickland reaction, where the coupled oxidation and reduction of these amino acids generate ATP and NAD+, respectively [29]. In the presence of proline and glycine, proline reductase (PR) and glycine reductase (GR), respectively are induced and reduce these amino acids. Among the two amino acids, *C. difficile* preferentially utilizes proline for NAD+ regeneration [29]. PrdR is a proline-responsive regulator that mediates proline-dependent PR activation and proline-dependent toxin gene suppression by an unknown mechanism (Figure 1) [29]. Analysis of the transcriptome of a PrdR mutant revealed that PrdR controls numerous additional reductive pathways, including glycine reductase, suggesting the direct regulator might be Rex, a sensor of redox status [28]. Rex is a DNA-binding protein that recognizes changes in the redox state and is only active when the intracellular NADH/NAD+ ratio is low. According to the current model [28], PrdR is activated and promotes PR expression when proline levels are high. As a result, the NADH/NAD+ ratio is low, and Rex acts as a repressor of alternative NAD+ synthesis pathways. When proline becomes scarce, on the other hand, the NADH/NAD+ ratio rises, and NADH inhibits Rex-dependent suppression of alternative pathways. When NAD+ is regenerated via these alternative reductive pathways, accumulation of butyrate occurs and stimulates toxin synthesis.

Role of c-di-GMP in toxin gene regulation

The signaling molecule cyclic di-guanosyl-5′monophosphate (c-di-GMP) is a second messenger in bacterial systems [35]. An increase in intracellular c-di-GMP concentration by ectopic expression of a c-di-GMP synthesis gene, *dccA*, has been shown to decrease the expression of toxin genes [33,36]. c-di-GMP has also been shown to negatively regulate the *flgB* flagellar operon by binding to the upstream riboswitch [36]. Along with flagellar genes, *flgB* operon also contains *sigD*, which positively regulates *tcdR* expression [20,21]. Therefore, c-di-GMP mediated regulation of toxin production is indirect and is mediated by SigD. It is important to note that a DNA inversion upstream of *flgB,* switch ON or OFF the expression of flagellar operon, including *sigD*. Thus, the phase variable expression of *sigD,* indirectly brings the toxin gene expression under the *flgB* regulatory switch [37]. In addition to SigD, other flagellar components, such as the flagellin FliC and FliD, also influence toxin production through unknown mechanisms [38,39].

Link between toxin production and sporulation

In sporulating bacteria, a complex regulatory network controls the transition between the stationary phase and the onset of sporulation. These regulatory mechanisms were extensively studied in *Bacillus subtilis*, where repressors such as AbrB, CodY, and SinR prevent the expression of genes that are expressed only during stationary phase or sporulation [40,41]. In *B. subtilis*, Spo0A is the master regulator that initiates sporulation after being phosphorylated through a phosphorelay system [40]. SigH, an alternative sigma factor, directs transcription of *spo0A* and the genes involved in the phosphorelay system [40]. *C. difficile* does not have orthologs of AbrB or the Spo0A-phosphorelay components, although it does have Spo0A, SigH, and SinR. At the onset of the stationary phase, SigH and Spo0A control toxin gene expression (Figure 1). In a *sigH* mutant, toxin genes and *tcdR* are overexpressed, implying that SigH represses toxin expression [42]. Spo0A effect on toxin gene expression was strain specific. In ribotype 027 strains, Spo0A acted as a repressor of toxin gene expression but had minimal impact in strains of ribotype 078 [43,44]. While it was clear that SigH and Spo0A indirectly affect toxin gene expression, the regulators involved were unknown. Results from the analysis of *sin* locus mutant suggested that SinR could be the link that connects toxin production with sporulation. The *sin* (sporulation inhibitor) locus in *C. difficile* encodes two proteins, SinR and SinI, and controls many genes involved in sporulation, motility, and biofilm formation [45–47]. SinR is a DNA-binding protein that inhibits target gene transcription, and SinI has an antagonistic interaction with SinR and inhibits its function by binding directly to the SinR protein. In the absence of SinR and SinI, decreased toxin production was observed [46]. The expression of *sinR* alone was sufficient to complement this phenotype, suggesting SinR is a positive regulator of toxin synthesis. SinR influences toxin gene transcription by controlling the *sigD* expression [46]. Spo0A suppresses *sinR* expression by directly binding to its upstream DNA region [48]. In the absence of Spo0A (in the *spo0A* mutant and the *sigH* mutant), SinR is produced more, increasing the SigD production, leading to more toxin synthesis [42,48]. Other than Spo0A, CodY also regulates *sin* locus expression [46].

RstA is a newly discovered regulator that promotes sporulation initiation while inhibiting toxin production [49–51]. An earlier study suggested that RstA regulates toxin production

by repressing the transcription of SigD [50]. New data, however, indicates that this regulator binds to specific DNA sequences upstream of *tcdR* and controls its transcription directly [51]. The RstA is a member of the RNPP family of transcriptional regulators involved in quorum sensing [49]. While the link between RstA and quorum sensing is unknown, autoinducer (AI) like molecules have been found in *C. difficile*. All analyzed *C. difficile* genomes contain an incomplete *agr1*- locus that contains *agrDB*, which codes for components responsible for producing autoinducer peptides [52]. The two-component system *agrAC* that activates quorum sensing responsive genes in response to AI peptides is absent in the *agr-1* locus [52]. When the *agr-1* locus was deleted, the deletions strains produced no toxins, and the mutant was unable to colonize the mouse gut to initiate infection [53]. However, in a recent work, Ahmed *et. al* showed that deletion of *agrDB* locus in 630 Δ *erm* strain had little impact in toxin production, but accumulation of AgrD1 peptide in the cytoplasm in the absence of AgrB1 resulted in increased toxin gene transcription [54]. In addition to *agr1* locus, *C. difficile* strains belonging to ribotype 027 carry a complete *agrABCD* operon (*agr-2* locus) [52]. In strain R20291 (ribotype 027), the inactivation of *agrA* results in decreased toxin production (Figure 1) [53,55]. AgrA regulates the flagellar synthesis and the expression of genes involved in c-di-GMP metabolism [55], suggesting that AgrA's impact on toxin gene transcription is indirect and mediated via SigD. While these initial studies highlight the importance of *agr* loci in *C. difficile*, questions regarding their mechanisms of action remain unanswered and warrant further studies. In addition to the *agr* system, *C. difficile* has been found to include the LuxS gene, which is known to produce a class of quorum sensing molecules known as AI-2 [56]. When added to the culture medium early during growth, AI-2 up-regulates the expression of PaLoc genes through an unknown mechanism.

Other regulators controlling toxin production

LexA is one of the major players in the bacterial SOS regulatory network. Absence of LexA influences the *tcdA* transcription negatively. Even though the interaction of LexA with *tcdA* promoter was not experimentally tested, potential LexA binding motifs were identified in the *tcdA* promoter region [57,58]. This might explain why antibiotics that are known to trigger SOS responses, such as levofloxacin and ciprofloxacin, enhanced toxin A production when added at sub-inhibitory doses [57,58]. Similar to LexA, Mfd is also a highly conserved protein and functions in the nucleotide excision repair pathway in response to DNA damage and is known to affect transcription elongation by removing the stalled RNA polymerases [59]. In *C. difficile*, inactivation of *mfd* resulted in enhanced transcription of *tcdA* and *tcdB* [59]. It was hypothesized that Mfd might affect the transcriptional regulation of toxin genes by relieving RNA polymerase stalled at roadblocks created by CodY and/or CcpA, the two major repressors of toxin gene expression. More recently, CelR, the repressor of cellobiose utilization operon was identified and characterized [60]. In the absence of CelR, *C. difficile* produced more toxins than in its presence. Potential CelR binding sites were identified in genes responsible for c-di-GMP turnover, indicating the effect of CelR on toxin gene transcription is indirect, possibly through the activation of SigD [60].

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Highlights

- During infection, *Clostridioides difficile* produces the toxins TcdA and TcdB, which are directly linked to the severity of the disease.
- Toxin gene expression is influenced by a number of positive and negative regulators in response to environmental cues.
- Many of the same regulators that govern toxin gene expression also affect sporulation, biofilm formation, and motility.
- Our present understanding of toxin gene regulation by various regulators is summarized in this review.

Concluding remarks

Toxin gene expression in *C. difficile* is activated by a variety of environmental stimuli and regulated by several global regulators, demonstrating the intricacy of this regulatory network. Deeper insight into toxin genes regulations can help combat the rise in CDI incidence and may help identify alternative treatment strategies.

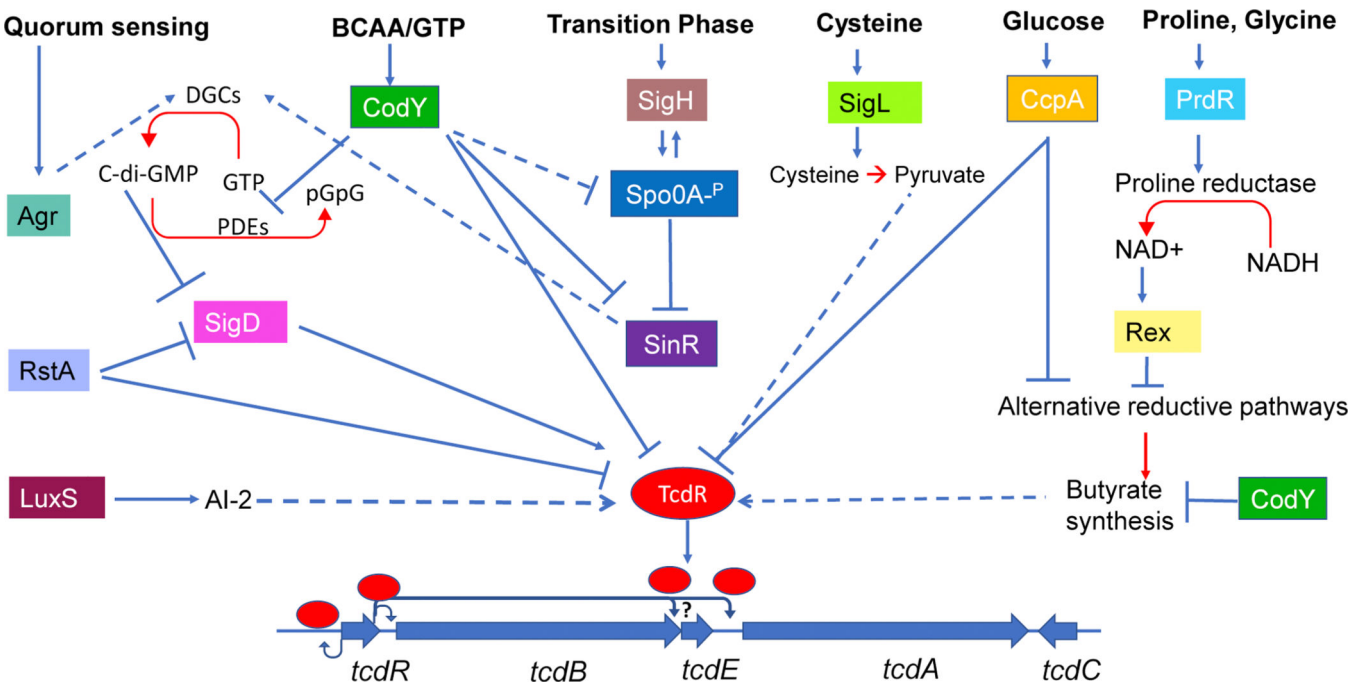

Figure 1. Toxin gene regulatory network in response to various stimuli.

TcdR activates the expression of its gene (*tcdR*) and both toxin genes *tcdA* and *tcdB*, while SigD activates the expression of *tcdR*. The repressors CodY, CcpA, and RstA bind to the promoter-regulatory regions at *tcdR*. CodY and CcpA also repress *tcdA*, *tcdB* directly by binding to their promoter region (not shown). All the other regulatory proteins influence toxin gene transcription indirectly, by regulating *tcdR* expression. Blue arrowed lines indicate positive controls, while lines ending with a bar across correspond to negative controls. Blue dashed arrows indicate mechanisms that are not fully understood. Enzymatic reactions are marked in red arrows. Abbreviations used: AI-2 (Auto inducers), GTP (Guanosine triphosphate), pGpG (5′-phosphoguanylyl-(3′−5′)-guanosine), c-di-GMP (cyclic di-guanosyl-5′monophosphate), BCCAs (branch chain amino acids), NAD (Nicotinamide adenine dinucleotide), DGCs (Di-Guanylate cyclases), PDEs (Phosphodiesterases).