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Integration of metabolism and virulence in *Clostridium difficile*

Laurent Bouillaut^a, Thomas Dubois^b, Abraham L. Sonenshein^{a,*}, and Bruno Dupuy^b

^aDepartment of Molecular Biology and Microbiology, Tufts University School of Medicine, 136 Harrison Avenue, Boston, MA 02111 USA

^bLaboratoire Pathogénèse des Bactéries Anaérobies, Institut Pasteur, 25 rue du Dr Roux, 75724 Paris France

Abstract

Synthesis of the major toxin proteins of the diarrheal pathogen, *Clostridium difficile*, is dependent on the activity of TcdR, an initiation (sigma) factor of RNA polymerase. The synthesis of TcdR and the activation of toxin gene expression are responsive to multiple components in the bacterium's nutritional environment, such as the presence of certain sugars, amino acids, and fatty acids. This review summarizes current knowledge about the mechanisms responsible for repression of toxin synthesis when glucose or branched-chain amino acids or proline are in excess and the pathways that lead to synthesis of butyrate, an activator of toxin synthesis. The regulatory proteins implicated in these mechanisms also play key roles in modulating bacterial metabolic pathways, suggesting that *C. difficile* pathogenesis is intimately connected to the bacterium's metabolic state.

Keywords

Clostridium difficile; toxinogenesis; metabolic regulation; redox state; Stickland metabolism

Introduction

The ability to adapt efficiently to a wide range of environmental changes, especially to alterations in nutritional quality, is a universal trait in the bacterial world. In many pathogenic bacteria, adaptation to an altered environment is often associated with the activation of the transcription of virulence genes; hence, many virulence genes are regulated by environmental and nutritional signals suggesting a tight link between metabolism and virulence.

In *Clostridium difficile*, production of the two large enterotoxins (TcdA and TcdB), considered to be the primary virulence factors [1, 2], responds to various environmental

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laurent.bouillaut@tufts.edu. tdubois@pasteur.fr. (Correspondence and reprints) linc.sonenshein@tufts.edu. bdupuy@pasteur.fr.

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conditions (at least when tested in the laboratory), including the availability of specific nutrients, temperature changes, and alteration of the redox potential [3-5]. For instance, when cells are grown in rich medium, the toxin genes are transcribed only when the cells enter stationary phase, i.e., when some form of nutrient limitation or accumulation of growth inhibiting substances occurs [6, 7]. In addition, the presence of a rapidly metabolizable carbon source [6, 8] or certain amino acids [9, 10] inhibits toxin gene expression. Once expression of the toxin genes is induced, the toxin proteins accumulate inside the cell and are slowly released over the course of several hours. Table 1 summarizes the environmental factors found to affect toxin production.

Some of the molecular mechanisms regulating *C. difficile* toxin gene expression in response to environmental signals have been elucidated [3, 6, 11-14]. Interestingly, several global regulators of metabolic pathways also regulate toxin gene expression; such co-regulation implies strongly that, from the bacterium's point of view, expression of some virulence genes serves a purpose that needs to be coordinated with expression of specific pathways. This review summarizes the roles of four regulatory proteins (CcpA, CodY, PrdR, and Rex) that couple the control of metabolism and pathogenesis in response to the presence of specific nutrients. In addition, toxin gene expression is known to respond to proteins (e.g., sigma-H and Spo0A) that play essential roles in regulation of spore formation, another nutrient-sensitive process. Thus, *C. difficile* has evolved to place the expression of its major virulence factors under the control of a panel of nutrient-sensing regulators that act in cooperative or antagonistic ways to cause the appropriate amount of toxin synthesis under a variety of environmental conditions.

The Pathogenicity Locus (PaLoc)

The genes encoding the two major toxins TcdA (*tcdA*) and TcdB (*tcdB*) are located within a 19.6 kb pathogenicity locus (PaLoc) (Fig. 1) found only in toxigenic strains of *C. difficile* [15]. This locus also includes genes encoding an RNA polymerase sigma factor (TcdR) that is essential for toxin gene expression [16], an antagonist of TcdR (named TcdC) [17] and a holin-like protein, TcdE, that is required for efficient release of TcdA and TcdB from the bacteria [18]. Although the PaLoc has some characteristics of a mobile genetic element, it does not appear to be intrinsically mobile and is located at the same site in all toxigenic *C. difficile* strains [15]. Nevertheless, given that the base composition of the PaLoc differs from that of the genome as a whole, it is likely that the PaLoc was acquired by horizontal transfer. Consistent with this hypothesis, the PaLoc was shown to be transferable by a presumably unlinked conjugation-like mechanism to a non-toxigenic strain, resulting in its conversion to a toxin producer [19]. Variations in the PaLoc region, including insertions, deletions and point mutations, have been used to define 32 toxinotypes among *C. difficile* isolates in comparison to the reference strain VPI10463 [20].

When *C. difficile* cells are grown in rich medium, the *tcdR*, *tcdB*, *tcdE* and *tcdA* genes are induced in stationary phase. Although *tcdA* and *tcdB* are transcribed mainly from their own promoters [6, 7], their transcripts can be found in both polycistronic and monocistronic mRNAs [7]. The promoter regions of *tcdA* and *tcdB* do not resemble the canonical σ^{70}

consensus promoters of prokaryotes but show strong similarities to each other and to certain other toxin and bacteriocin gene promoters in several *Clostridium* species [6, 7, 21].

Toxin gene transcription is activated by multiple sigma factors

The *tcdR* gene (previously named *tcdD* or *txeR*) encodes a basic protein of 22 kDa [15, 22]. Moncrief *et al.* provided the first evidence that TcdR is a positive regulator of the *C. difficile* toxin genes when they successfully activated *tcdA* and *tcdB* reporter fusions by expressing TcdR *in trans* in *Escherichia coli* [22]. Similar effects were seen in *C. perfringens* [16] and *C. difficile* [23]. Mani & Dupuy provided the genetic and biochemical evidence that TcdR is required for specific transcription from the *tcdA* and *tcdB* promoters and that TcdR functions specifically as a sigma factor of RNA polymerase (RNAP) [16]. TcdR also activates its own expression [23], consistent with the presence in the region upstream of the *tcdR* gene of two potential promoters for TcdR-dependent transcription (Fig. 2) [23]. Recently, El Meouche *et al.* identified a SigD-dependent promoter sequence upstream of *tcdR* (Fig. 2) [24]. Moreover, they showed that SigD, a sigma factor usually associated with motility gene expression, positively controls toxin gene expression [24].

TcdR is a member of a discrete group (Group V) of σ factors within the σ^{70} family. Other members of Group V include UviA, which directs bacteriocin gene transcription in *C. perfringens*, BotR and TetR, which activate transcription of the botulinum and tetanus toxin genes, respectively [25], TcsR, which stimulates transcription of the lethal and hemorrhagic toxin genes of *Clostridium sordellii* [26] and TpeR, the activator of TpeL cytotoxin synthesis in *C. perfringens* [27]. Interestingly, all of these σ factors are encoded within genetic elements that might have been acquired by horizontal gene transfer. UviA and TetR are encoded on plasmids [28, 29], BotR within bacteriophage genomes in some *C. botulinum* strains [30], TpeR within an uncharacterized pathogenicity locus [27] and TcsR within a toxin locus that shows signatures of integrative and conjugative elements [26]. The organization of the sigma factor gene and its target gene promoters has also been largely conserved [26, 31]. Moreover, in all cases examined, the sigma factor gene is autoregulated and induced by one or more environmental stresses [26, 31]. Additional homologues of the group V sigma factors can be found in other pathogenic *Clostridium* spp., such as *Clostridium novyi* (G. Carter and D. Lyras, personal communication), as well as in non-pathogenic *Clostridium* spp., such as *C. acetobutylicum* [31]. The functions of these latter sigma factor homologs remain to be determined.

A negative role for TcdC in toxin expression was first suggested based on the inverse orientation and timing of its expression relative to those of the other members of the PaLoc [7]. This putative role of TcdC in toxin regulation appeared to be reinforced by the emergence of epidemic strains that carry deletions or frameshift mutations in the *tcdC* gene and produce high levels of both toxins [32, 33].

TcdC is an acidic, membrane-associated protein with a predicted molecular weight of 26-kDa [15, 34]. TcdC can form dimers, which is consistent with the presence of a coiled-coil motif in the middle of the protein, and appears to act as an anti-sigma factor [17, 35]. TcdC negatively regulates *C. difficile* toxin gene expression by interfering with the ability of the

TcdR-containing RNAP to recognize the *tcdA* and *tcdB* promoters [17]. In fact, both free TcdR and TcdR-containing holoenzyme are sensitive to TcdC. Once a stable open complex is formed with the *tcdA* promoter, TcdC cannot disrupt the binding of RNAP to the promoter [17]. Recently, van Leeuwen *et al.* [35] showed that TcdC could bind to DNA folded into G-quadruplex structures containing repetitive guanine nucleotides, suggesting that TcdC might also act by destabilizing open complex formation before transcription initiation. However, no quadruplex-forming motif with multiple G-stretches is found in the PaLoc [35]. In support of a direct contact between TcdR and TcdC, TcdC was shown to inhibit transcription dependent on alternative sigma factors such as UviA and SigW, but not primary SigA-type factors [17]. This strongly suggests that the mode of action of TcdC is more dependent on the nature of the sigma factor than of the core enzyme. Nevertheless, questions still remain as to how TcdC inactivates or destabilizes the TcdR-containing RNAP holoenzyme.

Although blocking of TcdR-dependent transcription by TcdC *in vitro* is not in dispute, different experimental approaches have led to conflicting conclusions about the importance of TcdC in regulating toxin expression in living cells. The prevailing model that TcdC is a negative regulator of toxin expression was supported by the finding that introduction of a functional *tcdC* gene into an epidemic strain that carries a non-functional *tcdC* gene (M7404, a PCR ribotype NAP1/027 strain) resulted in decreased toxin production and attenuated virulence in a hamster model [36]. However, chromosomal complementation in strain R20291, another PCR ribotype NAP1/027 strain with an inactive *tcdC* gene, resulted in no discernible effect on toxin expression [37]. Moreover, other studies showed that disruption of the *tcdC* gene in strain 630 Erm had little if any effect on toxin expression under the conditions tested [37, 38]. The reasons for the conflicting data may relate to experimental variations, including the strain used or the specific growth conditions, either of which might affect the level of TcdC expression or activity.

Given the complexity of toxin gene expression, the various components of the environment that affect toxin synthesis (Table 1) could do so by altering expression or activity of TcdR, SigD, TcdC, TcdE or the toxin proteins themselves.

Roles of proline and glycine and regulation of Stickland metabolism by PrdR and Rex

In the mid-1930's, using extracts of *Clostridium sporogenes*, Stickland and Woods discovered coupled pathways of amino acid metabolism in which oxidative deamination and decarboxylation of one amino acid, generating ATP and NADH, is linked to reduction of D-proline or reductive deamination of glycine [39-41]. The products of D-proline reductase, the proline metabolism complex, [42] are 5-aminovalerate and NAD⁺; the products of glycine reductase (GR) are ammonium ion, acetate and ATP. *C. difficile* PR and GR are seleno-enzyme complexes [43] encoded by distinct multi-gene clusters. Jackson *et al.* [43] suggested that, given the selenium requirement of *C. difficile* for growth in a defined, basal medium, the selenium-containing reductases (PR and GR) are likely to play a key role in the physiology of the bacteria. In fact, both glycine and L-proline are required for optimal growth in defined media [44-46] and PR is required for optimal growth even in a rich,

complex medium containing a multitude of carbon and nitrogen sources [47]. Interestingly, the lack of GR does not affect growth in rich medium and addition of proline to the medium increases expression of PR but decreases expression of GR [43]. The observations that proline and/or a mixture of seven amino acids containing glycine and proline strongly decrease *C. difficile* toxin yield [3, 10] suggested a role for Stickland metabolism in the regulation of toxin production.

PrdR, an apparent sigma-54-dependent transcriptional activator encoded by a gene located upstream of the PR gene cluster [43, 47], is the mediator of proline-dependent activation of the expression of PR and proline-dependent repression of the toxin gene *tcdA* and the GR operon during growth *in vitro* [47]. Interestingly, Janoir *et al.* also observed inverse regulation of the *prd* and *grd* gene clusters during the course of infection of monoxenic mice [48]. Broader effects of proline on *C. difficile* gene expression were revealed by microarray analysis. These effects are dependent on PrdR and mostly affect reductive pathways (L. Bouillaut, T. Dubois, M. Monot, B. Dupuy and A. L. Sonenshein, manuscript in preparation). If proline is limiting in the growth medium or if PR or PrdR is inactive, alternative pathways for NAD⁺ regeneration [e.g., glycine reductase, alcohol dehydrogenase, butyrate production from acetyl CoA and succinate] (Fig. 3) are induced (Bouillaut *et al.*, manuscript in preparation). This phenomenon implies a global role for PrdR in the control of reductive pathways. However, is this control direct? Given that a functional PR is required in addition to PrdR for proline-dependent repression of GR and other reductive pathways [47], it seemed possible that such a broad pattern of repression might not be mediated directly by PrdR. Since all of these pathways regenerate NAD⁺ from NADH, the direct regulator might be a sensor of redox status.

Several Gram-positive bacteria (e.g., *C. acetobutylicum* [49], *Streptomyces coelicolor* [50], *Thermus aquaticus* [51], *B. subtilis* [52, 53], and *S. aureus* [42]) have a redox-dependent transcriptional repressor (Rex) that plays a key role in regulating anaerobic metabolism. Rex is a DNA binding protein containing a Rossmann fold dinucleotide binding site [51]. Structural studies of *T. aquaticus* Rex-NADH/NAD⁺ interactions have shown that binding of NADH induces a conformational change as a result of which binding of Rex to its DNA target site (TTGTGAA[a/t]₆TTCACAA) is blocked [51]. In other words, Rex is only active as a DNA-binding protein when the intracellular NADH/NAD⁺ ratio is low [50]. Rex is found in *C. difficile* and putative binding sites can be identified upstream of genes involved in alternative pathways for NAD⁺ regeneration [54]. In fact, a *rex* null mutant of *C. difficile* proved to be insensitive to the presence of proline in the medium and to be defective in repression of the GR locus, alcohol dehydrogenase, the acetyl CoA-to-butyrate pathway and the succinate-to-butyrate pathway (Fig. 3). In accord with the general model, binding of *C. difficile* Rex protein to its target sites was stimulated by NAD⁺, but inhibited by NADH [Bouillaut *et al.*, manuscript in preparation]. Since *C. difficile* Rex acts as a repressor of all of its target genes identified to date, Rex dissociates from the operator sites when intracellular NADH concentrations increase relative to NAD⁺ concentrations, allowing expression of the genes it represses. In addition, because the intracellular concentration of NADH is about 10-fold lower than that of NAD⁺ and the affinity of Rex for NADH is 10-

fold higher than for NAD⁺, Rex may allow a sensitive control of the expression of NAD⁺ - regenerating pathways.

Our model for how proline and PrdR influence the activity of Rex and hence the transcription of Rex-repressed genes is the following. Reduction of proline is the most efficient and, therefore, the favored pathway for NAD⁺ regeneration. (This hypothesis is supported by the fact that PR gene expression is also activated by glucose (see below); glycolysis is a major producer of NADH.) When proline is in excess, PrdR is active and stimulates PR expression; as a result, enough NADH is oxidized to satisfy the cell's requirements. In the presence of a low ratio of NADH to NAD⁺, Rex is active as a repressor of the alternative NAD⁺ regeneration pathways. When proline becomes limiting and the NADH/NAD⁺ ratio rises, Rex loses activity as a repressor and the alternative pathways are expressed (Fig. 3). Regeneration of NAD⁺ using these alternative pathways leads to the production of butyrate, a stimulator of toxin synthesis. Therefore, it is tempting to think that butyrate production signals redox stress and nutrient limitation (i.e., proline), as a result of which the cell is programmed to activate toxin synthesis. The molecular mechanism by which butyrate activates toxin synthesis is unknown.

Repression of toxin synthesis by glucose is mediated by CcpA

In complex media, the presence of glucose or other rapidly metabolizable carbon sources, such as fructose or mannitol, leads to a strong decrease of toxin production [6, 10]. The inhibitory effect of glucose is not due to accumulation of acidic metabolites derived from glucose metabolism [6, 10] and was observed in multiple *C. difficile* strains [6]. Furthermore, the repressive effect of glucose on toxin gene expression occurs at the transcriptional level, consistent with the repression by glucose of *tcdR* expression [6, 23].

The effects of rapidly metabolizable sugars on toxin gene expression are examples of carbon catabolite repression (CCR). CCR in bacteria has two principal elements: inducer exclusion, usually mediated by a component of the glucose transporter, and transcriptional regulation by a glucose-responsive DNA binding protein. In low G+C Gram-positive bacteria, the major agent of CCR is CcpA, a protein of the Lac repressor family [55]. CcpA usually acts as a repressor of utilization of alternative carbon sources and as a positive regulator of pathways associated with glycolysis, such as carbon overflow pathways. In most Gram-positive bacteria studied to date, the HPr protein, a component of the phosphoenolpyruvate-dependent carbohydrate:phosphotransferase system (PTS), is involved in both sugar uptake and the regulatory activity of CcpA [56]. To activate CcpA, HPr must be phosphorylated at serine-46 by HPr kinase/phosphorylase, an enzyme whose activity is stimulated by fructose-1,6-bisphosphate. The CcpA/HPr~P complex then binds to catabolite responsive elements (*cre* sites) of target genes to affect their expression. Few of these details of CcpA-mediated regulation of carbon metabolism have been established for *C. difficile*, but the role of CcpA in toxin gene regulation is now well known [11, 57]. That is, no glucose-dependent repression of toxin gene expression was observed in mutant strains defective in genes of the PTS pathway or in *ccpA*. However, mutations in *hprK*, the gene for HPr kinase/phosphorylase, do not affect repression of toxin gene expression [11]. CcpA mediates repression of toxin production *in vitro* by interacting directly with the promoter regions of

the PaLoc with the strongest affinity for the promoter region of *tcdR* (Fig. 2) [57]. The sequence of the CcpA-binding site defined for *C. difficile* is significantly different than the consensus *cre* site defined in *B. subtilis* [57]. Interestingly, although toxin production in the *C. difficile*, *ccpA* mutant strain is constitutive with respect to glucose, the level of expression is lower than in the wild-type strain grown without glucose [11]. Moreover, in a glucose-limited defined medium (SDM), toxin production is low compared to high-glucose SDM medium [10], implying that glucose limitation does not induce toxin production. Thus, it appears that one or more other regulators controlled by CcpA might contribute to toxin gene regulation independently of the presence of glucose. In fact, CcpA controls directly or indirectly a large number of regulatory proteins [57] and participates with other global regulators like CodY and Rex (see below) in coordinating metabolism and virulence. As described below, toxin synthesis is induced in the presence of butyric acid and the genes whose products produce butyrate from succinate or acetyl-CoA are regulated by CcpA, CodY and Rex (Fig. 3) [57, 58].

Repression of toxin synthesis by a mixture of amino acids is mediated by CodY

CodY is a global regulator involved in the adaptive response to nutrient limitation in low G +C content Gram-positive bacteria [59]. First, identified as a repressor of the *B. subtilis* dipeptide permease operon [60], CodY is now known to control hundreds of genes involved in major physiological processes in non-pathogenic (*Bacillus* [61], *Lactococcus* [62]) and pathogenic bacteria (*Listeria* [63]), *Streptococcus* [64, 65], *Bacillus* [66, 67] and *Staphylococcus* [68, 69]). In *C. difficile*, 146 genes putatively organized in 82 transcription units are overexpressed at least 4-fold in a *codY* mutant and 19 genes (15 transcription units) are underexpressed [58]. Genome-wide identification of direct CodY targets suggests that CodY controls 52 of the 82 transcription units directly [58]; most target genes are involved in biosynthesis of amino acids, nutrient transport, fermentation pathways, toxin production and spore formation.

Structural studies of *B. subtilis* CodY revealed that the protein contains two functional domains linked by a rigid α -helical domain [70]. A winged helix-turn-helix domain needed for binding to DNA lies near the C-terminus, and a GAF domain that binds BCAAs is close to the N-terminus [70, 71]. *In vitro*, the DNA-binding activity of CodY is enhanced by interaction with two types of effectors, branched-chain amino acids (isoleucine, leucine and valine; BCAAs) [71, 72] and GTP [73, 74]. The effects of BCAAs and GTP on CodY affinity for DNA are synergistic [74]. CodY binding sites in all bacteria examined to date are variants of the consensus motif AATTTTCWGAAAATT [64, 75, 76]. The intracellular levels of BCAAs and GTP decrease when nutrients become limiting [77, 78], triggering the release of CodY from its target sites and altering expression of CodY-responsive genes.

The role of BCAAs in activating CodY can explain why isoleucine [79] and a seven-amino acid mixture that includes the BCAAs [10] suppress *C. difficile* toxin production. In addition, the GTP pool reflects the availability of all amino acids, since, when any amino acid is limiting, the stringent response is induced and the GTP pool drops significantly [80]. In fact, CodY was the first regulator encoded outside the PaLoc found to be involved in

toxin regulation. All the genes of the PaLoc, i.e., *tcdR*, *tcdB*, *tcdA*, *tcdE* and *tcdC* are derepressed in a *C. difficile* *codY* mutant [14, 58]. The amount of toxin A protein released into the culture fluid after 24 hrs of growth is 100-fold higher in the *codY* mutant strain than in its parent (L. Bouillaut, X. Sun, S. Tzipori and A. L. Sonenshein, unpublished). CodY regulates toxin gene expression by binding to the *tcdR* promoter region at three different locations with varying affinities (Fig. 2); two of the binding sites overlap with sigma factor-binding regions.

In addition to its direct effect on expression of the toxin gene locus, CodY contributes to repression of toxin synthesis by regulating the synthesis of butyrate. All the transcription units required for butyrate synthesis from acetyl CoA and succinate (Fig. 3) are direct CodY targets [58]. Thus, just as Rex represses most of the same genes in response to the ratio of NAD⁺ to NADH, CodY contributes to repression when BCAAs and GTP are in excess.

Role of sporulation regulators SigH and Spo0A

During transition from exponential growth phase to stationary phase, complex regulatory circuits are activated to modify metabolism and turn on both toxin synthesis and sporulation. Initiation of spore formation in *B. subtilis* is controlled by a regulatory cascade consisting of five kinases, two phosphorylated intermediate proteins (Spo0F and Spo0B) and several phosphatases [81]. The end result is the finely tuned activation-by-phosphorylation of Spo0A, a response regulator that activates transcription of several key sporulation genes as well as other stationary phase genes and represses the expression of inhibitors of sporulation. Incompletely defined environmental signals activate the phosphorelay [81]. *C. difficile* encodes a Spo0A homolog, but does not encode homologs of Spo0F or Spo0B; three orphan histidine kinases are the only factors known to modulate Spo0A activity [12, 13, 82]. As in *B. subtilis*, not all *C. difficile* genes affected by Spo0A activation are involved in sporulation. *C. difficile* Spo0A also contributes to regulation of metabolism and, in some strains, to toxin production. Indeed, glucose uptake, glycolysis and butyrate production (Fig. 3) are underexpressed in a *C. difficile* *spo0A* mutant [83]. These effects of a *spo0A* mutant appear to be strain-specific. Spo0A represses toxin gene expression in two ribotype 027 strains but has no or marginal effects in ribotype 078 and 012 strains [83-86]. The mechanism of the putative regulation of the toxin locus by Spo0A remains to be determined and is likely indirect, since no Spo0A binding sequences have been found upstream of any of the PaLoc genes [12].

Another key element of the transition phase and early sporulation events is the alternative sigma factor, SigH. In *C. difficile*, SigH is required for expression of *spo0A*, the genes whose products phosphorylate Spo0A, the butyrate biosynthesis pathway and virulence [13]. Interestingly, the regulation of SigH and Spo0A is reciprocal; SigH modulates *spo0A* transcription and Spo0A activates *sigH* expression. The effects of SigH and Spo0A on toxin gene expression may be different, however. That is, in a *sigH* mutant, the *tcdR*, *tcdA* and *tcdB* genes are overexpressed, implying that SigH controls expression of a gene whose product inhibits toxin gene expression [13]. The mechanistic basis for this apparent inhibition is not known.

Future directions

The complex, environmentally-responsive manner in which *C. difficile* controls expression of its major virulence factors suggests that the bacterium's strategy for causing damage to the host is closely tied to its nutritional status (Fig. 4). The use by *C. difficile* of global metabolic regulators, such as CcpA, CodY and Rex, to control toxin gene expression implies that, from the bacterium's point of view, virulence is to a great extent a mechanism for improving nutrient availability.

The specific metabolites that influence the activities of these regulators can be viewed as indicators of the viability of major metabolic pathways. For instance, CcpA is activated by fructose-1,6-bisphosphate, a key substrate of glycolysis, CodY responds to GTP (an indicator of ability to make RNA and protein) and BCAAs (indicators of ability to make protein and membrane fatty acids), Rex responds to the NADH/NAD⁺ ratio (an indicator of redox state) and PrdR responds to proline, the substrate of the primary pathway for NAD⁺ regeneration (Fig. 4). The mechanisms by which *C. difficile* responds to cysteine, biotin and butyrate remain to be discovered. The role of butyrate in regulating toxin synthesis is of great potential interest. First, *C. difficile* appears to produce butyrate only when the cells have an excess of NADH, suggesting that stimulation of toxin synthesis by butyrate is a response to a high NADH/NAD⁺ ratio. Second, butyrate is produced by many inhabitants of the GI tract, suggesting that *C. difficile* turns on toxin synthesis when in the presence of other butyrate-producing species. The latter hypothesis would seem to run counter to the presumption that *C. difficile* is only able to colonize the colon when the normal microbiota has been compromised. Finally, butyryl CoA, the precursor of butyrate, is also the precursor of butanol, an inhibitor of toxin synthesis (Figs. 3, 4) [8]. Discovering how the bacterium decides whether to convert butyryl CoA to butanol or to butyrate will reveal an important decision point for *C. difficile* and would potentially yield new methods of reducing virulence.

For an intestinal pathogen, such as *C. difficile*, inducing diarrhea is a mechanism for dispersal of the bacteria to new and potentially more supportive environments. Given that it is only the spore form of *C. difficile* that can survive outside the colon, however, the dispersed bacterial population must have a substantial spore component in order to maintain and spread the organism. This limitation on *C. difficile* survival would seem to demand that the same population that causes diarrhea include a reasonable number of spore-forming cells. Such an obligation raises a critical unanswered question: Do the same cells in a population produce both toxins and spores or is the population split between toxin producers and spore-formers? The negative correlation between the activity of SigH and the ability of cells to produce toxin suggests that the two processes are antithetical and predicts that a given cell can produce either toxins or spores but not both. However, it is also possible that toxin synthesis and sporulation occur in different time frames, so that a cell might produce toxin before committing to form a spore.

In summary, virtually everything we know about the regulation of the *C. difficile* PaLoc indicates an intimate connection between metabolism and virulence (Fig. 4). As virulence factors other than the major toxin proteins become identified and verified, it will be

interesting to determine how their genes are regulated as well. The answers will tell us the extent to which *C. difficile* pathogenesis is determined by intracellular metabolite pools.

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Fig. 1.

Schematic diagram of PaLoc in toxigenic *C. difficile* strains. In non-toxigenic strains this region is replaced by a 115-bp sequence. Arrows indicate the positive regulation of *tcdR*, *tcdB* and *tcdA* by σ^{TcdR} . *cdu1* and *cdd1* are similar to genes encoding a transcriptional regulator and a hypothetical protein, respectively, and appear to be of bacteriophage origin. The question mark indicates that the role of TcdR in *tcdE* transcription is unclear. This figure previously appeared in ref. 18.

Fig. 2.

Schematic representation of the *tcdR* promoter region. The arrows indicate transcription start sites activated by σ^A -, σ^D - and TcdR-containing forms of RNA polymerase. Blue and pink boxes represent CodY and CcpA binding sites, respectively. Positions of sites are indicated relative to the ATG start codon.

Fig. 3.
Roles of global regulators in controlling expression of pathways for NAD⁺ regeneration.

Fig. 4.

Effects of metabolites and regulatory proteins on *C. difficile* toxin synthesis. The metabolites that activate each regulatory protein are indicated; FBP, fructose-1,6-bisphosphate. Question marks indicate that metabolites affect toxin synthesis via unknown proteins and that some regulators (PrdR, SigH, Spo0A) are likely to affect toxin gene expression indirectly.

Table 1

Environmental effectors of toxin synthesis.

Inhibitors	Activators
Glucose [6, 10]	Butyrate [8]
Mixture of Gly, Met, Trp, Thr, Ile, Leu, Val [8]	Temperature (37°C) [3]
Proline [8, 47]	
Butanol [8]	
Cysteine [8]	
Biotin [4, 10]	