Review

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Clostridium difficile toxin synthesis is negatively regulated by TcdC

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Clostridium difficile toxin synthesis is growth phase-dependent and is regulated by various environmental signals. The toxin genes *tcdA* and *tcdB* are located in a pathogenicity locus, which also includes three accessory genes, *tcdR*, *tcdC* and *tcdE*. TcdR has been shown to act as an alternative σ factor that mediates positive regulation of both the toxin genes and its own gene. The *tcdA*, *tcdB* and *tcdR* genes are transcribed during the stationary growth phase. The *tcdC* gene, however, is expressed during exponential phase. This expression pattern suggested that TcdC may act as a negative regulator of toxin gene expression. TcdC is a small acidic protein without any conserved DNA-binding motif. It is able to form dimers and its N-terminal region includes a putative transmembrane domain. Genetic and biochemical evidence showed that TcdC negatively regulates *C. difficile* toxin synthesis by interfering with the ability of TcdR-containing RNA polymerase to recognize the *tcdA* and *tcdB* promoters. In addition, the *C. difficile* NAP1/027 epidemic strains that produce higher levels of toxins have mutations in *tcdC*. Interestingly, a frameshift mutation at position 117 of the *tcdC* coding sequence seems to be, at least in part, responsible for the hypervirulence phenotype of these epidemic strains.

Introduction

A relationship between the symptoms of antibiotic-associated diarrhoea and the toxigenic potential of the *Clostridium difficile* strain responsible for infection was suggested 20 years ago (Wren *et al.*, 1987). Akerlund's group recently confirmed this idea by showing that toxin levels are correlated with the severity of *C. difficile*-associated disease (CDAD) (Akerlund *et al.*, 2006). This idea has also been reinforced by the emergence of an epidemic strain, named *C. difficile* NAP1/027 (Warny *et al.*, 2005), which is responsible for a massive increase in disease incidence and associated death. In fact, the major characteristic of this strain is that it produces higher amounts of toxins A and B than those produced by non-epidemic strains. Taken together, these results underline the importance of the regulation of toxin synthesis in the pathogenicity process of *C. difficile*.

Several studies have shown that toxin production is sensitive to both bacterial and host factors (Akerlund *et al.*, 2006; Borriello *et al.*, 1987; Mahe *et al.*, 1987; McFarland *et al.*, 1991; Ward & Young, 1997). The age of the patient and levels of toxin-neutralizing antibodies are factors that affect severity of disease and the risk of relapse of CDAD (Kyne *et al.*, 2000, 2001; Lyster *et al.*, 1988; Warny *et al.*, 1994). In *in vitro* culture, variations in the production of both toxins are highly influenced by the nutrients present in the medium (Haslam *et al.*, 1986) and by environmental conditions. Toxin synthesis increases as

cells enter stationary phase (Hundsberger *et al.*, 1997; Dupuy & Sonenshein, 1998) and is controlled by growth temperature with an optimum at 37 °C (Karlsson *et al.*, 2003). In addition, toxin yield is affected by the presence in the growth medium of sulphur-containing amino acids, butyric acid and butanol (Karlsson *et al.*, 1999, 2000) and carbon sources such as glucose and most PTS sugars (Dupuy & Sonenshein, 1998). Moreover, addition of certain antibiotics (Onderdonk *et al.*, 1979; Nakamura *et al.*, 1982; Honda *et al.*, 1983) or limited levels of biotin (Yamakawa *et al.*, 1996) induces or stimulates toxin production, respectively.

Although inducing cues for toxin production and several implicated environmental factors have been studied in detail, the systems involved in these adaptive regulations are poorly understood. One breakthrough in elucidating the mechanism of toxin gene regulation came from the molecular analysis of the pathogenicity locus (PaLoc) of the virulent strains.

TcdR is the first PaLoc regulatory protein described that regulates toxin expression

The two toxin genes *tcdA* and *tcdB* and the genes encoding the proteins TcdR, TcdE and TcdC form a chromosomal pathogenicity locus (PaLoc; Fig. 1) (Braun *et al.*, 1996; von Eichel-Streiber *et al.*, 1992). The role of TcdE has not yet been completely clarified. The transport function of TcdE is based on its similarity to phage holin proteins and has

Fig. 1. Genetic organization of the *C. difficile* pathogenicity locus (PaLoc).

been proposed to be important for release of toxins into the environment (Tan *et al.*, 2001). In fact, preliminary results show that TcdE is able to complement an *Escherichia coli* λ phage deprived of its holin (B. Dupuy and M. Santos, unpublished data).

TcdR has weak similarities with transcriptional activators of several *Clostridium* species and with families of RNA polymerase σ factors found in many organisms (Moncrief *et al.*, 1997; Mani & Dupuy, 2001). In 1997, Moncrief *et al.* (1997), working with *E. coli*, provided the first evidence for a positive role of TcdR in toxin gene regulation. These results were confirmed by similar experiments using *Clostridium perfringens* as surrogate host (Mani & Dupuy, 2001) and later in *C. difficile* (Mani *et al.*, 2002). Genetic and biochemical evidence has shown that TcdR functions as an RNA polymerase σ factor that is indispensable for transcription initiation from the *tcdA* and *tcdB* promoters (Mani & Dupuy, 2001). TcdR also activates its own expression (Mani *et al.*, 2002).

Proteins similar to TcdR have been found in other pathogenic clostridia such as *Clostridium botulinum* (BotR), *Clostridium tetani* (TetR) and *C. perfringens* (UviA). All of these proteins act as alternative σ factors that are indispensable for the synthesis of botulinum and tetanus neurotoxins and a UV-inducible bacteriocin in *C. perfringens* (Dupuy *et al.*, 2005; Raffestin *et al.*, 2005). These clostridial σ factors differ enough in structure and function from other subgroups of the σ^{70} -family to be assigned to their own group (group 5).

TcdR expression is influenced by the medium composition, the growth temperature and the cellular growth phase, in the same way as expression of the toxin genes (Mani *et al.*, 2002; Karlsson *et al.*, 2003). Thus activation of TcdR expression or activity is likely to be the trigger for induction of toxin gene transcription. Interestingly, the same pattern of expression is observed for all genes of the PaLoc except for *tcdC*, which is mainly expressed during the rapid exponential growth phase, while its expression is shut off when cells enter stationary growth phase (Hundsberger *et al.*, 1997).

TcdC, a negative regulator of *C. difficile* toxin synthesis

This inverse transcription pattern suggested the possibility that TcdC plays a negative role in toxin regulation

(Hundsberger *et al.*, 1997). Moreover, the emergence of epidemic strains that produce high levels of toxins and carry deletions or frameshift mutations in the *tcdC* gene reinforced the putative negative role of TcdC in toxin regulation (MacCannell *et al.*, 2006; Warny *et al.*, 2005).

The regulatory role of TcdC was first tested *in vivo* by studying the behaviour of fusions of the *tcdA* gene promoter to the reporter gene *gusA*, which encodes *E. coli* β -glucuronidase (Matamouros *et al.*, 2007). Expression of TcdR *in trans* activated expression from the *tcdA* promoter. However, when TcdC was co-expressed with TcdR, activity was strongly reduced. This was the first direct evidence that TcdC negatively regulates *tcdA* transcription and stimulated the search for the mechanism by which TcdC negatively regulates toxin gene transcription.

TcdC is an acidic protein with a predicted molecular mass of 26 kDa (Braun *et al.*, 1996). Govind *et al.* (2006) have recently demonstrated that TcdC is a membrane-associated protein, which is in agreement with the predicted transmembrane domain found in the N-terminal region of the protein. In addition, TcdC forms dimers, which is consistent with the presence of a coiled-coil motif found in the middle of the protein (Matamouros *et al.*, 2007).

However, these biochemical characteristics did not really indicate how TcdC could work. Computational analysis of its sequence reveals that TcdC is not similar to any known regulatory protein and no DNA-binding motif has been identified. Based on the properties of TcdC, one of the hypotheses would be that TcdC could function as an anti- σ factor. Anti- σ factors are often transmembrane proteins that regulate gene expression by modulating the activity of a cognate σ factor (Brown & Hughes, 1995; Helmann, 1999). Nevertheless, there are several different mechanisms by which σ factors and anti- σ factors can interact (Minakhin & Severinov, 2005; Karlinsey *et al.*, 2000; Ades, 2004). In general, the basic principle is to sequester the cognate σ factor, thereby preventing the formation of an active RNA polymerase holoenzyme (RNAP). For example, under non-stressful conditions, RseA, the membrane-bound antagonist of *E. coli* σ^E , binds to σ^E and inhibits σ^E -directed transcription. However, under stressful conditions, degradation of RseA occurs within the membrane, releasing σ^E (Ades, 2004). Therefore, σ^E is free to form an active holoenzyme and activate transcription of σ^E -dependent promoters.

In *in vitro* run-off transcription assays, TcdR directs RNAP core enzyme to recognize and initiate transcription from the *tcdA* promoter. However, when TcdR is pre-incubated with TcdC before addition of the core RNAP, *tcdA* transcription is inhibited. No TcdC interaction with the *tcdA* promoter can be detected in gel mobility shift assays, which is consistent with the absence of any DNA-binding motif in the TcdC protein and indicates that TcdC must inhibit expression of toxin genes without interacting directly with their promoters. Instead, TcdC destabilizes the TcdR-containing holoenzyme bound to the *tcdA* promoter. That is, addition of TcdC during the reconstitution of the TcdR-containing holoenzyme decreases the affinity of the holoenzyme for the *tcdA* promoter. Thus TcdC seems to act by interfering with holoenzyme–P*tcdA* complex formation.

Both free TcdR and preformed TcdR-containing holoenzyme are sensitive to TcdC. In contrast, once a stable open complex is formed with the *tcdA* promoter, TcdC cannot disrupt the binding of the RNA polymerase to the promoter (Matamouros *et al.*, 2007), suggesting that TcdC acts at an early stage of transcription initiation.

During the initiation of the transcription, there are three major steps – the binding of the σ factor to the core RNAP, promoter recognition by the active holoenzyme and the formation of the open complex, followed by formation of the first phosphodiester bond (initiation) and the elongation process (Geszvain & Landick, 2005). Using surface plasmon resonance (SPR) analysis, we confirmed that TcdR binds to the core RNAP, as already shown (Mani & Dupuy, 2001), and that addition of TcdC decreases the association of TcdR with the core RNAP. Thus TcdC sequesters TcdR, in the manner of classical anti- σ factors (Helmann, 1999).

Interestingly, TcdC also interacts directly with core RNAP, a property not seen with other anti- σ factors. Thus we cannot rule out the possibility that TcdC, by directly interacting with the core RNAP, might compete with TcdR for binding to the core RNAP and thereby reduce the stability of TcdR–core complexes (Fig. 2).

Despite the fact that TcdC is able to retain TcdR in a pulldown experiment (Matamouros *et al.*, 2007), we were not able to show a direct interaction between TcdR and TcdC by SPR analysis. As a result, questions still remain as to how TcdC inactivates or destabilizes the TcdR-containing holoenzyme before open complex formation. In support of a direct contact between TcdR and TcdC, we showed that TcdC inhibits transcription directed by other alternative σ factors but is not able to repress transcription dependent on a primary σ factor (Matamouros *et al.*, 2007), implying that TcdC is specific to the alternative σ factors. Moreover, inhibition of transcription by TcdC is more dependent on the nature of the σ factor than on that of the core enzyme.

Role of TcdC in the epidemic strain NAP1/027

The recent and dramatic epidemic of CDAD reported in North America, Canada and Europe is due to the emergence of virulent *C. difficile* strains, characterized as toxinotype III PCR ribotype 027, that produce higher amounts of toxins than those produced by non-epidemic strains and, uniformly, carry the gene for the binary toxin (McDonald *et al.*, 2005; Pepin *et al.*, 2005; Warny *et al.*, 2005). Moreover, the epidemic strains have been described as having various deletions at the 3' end of the *tcdC* gene, resulting in truncated TcdC proteins. An 18 bp deletion

Fig. 2. Schematic representation of *C. difficile* toxin gene regulation by TcdC. When present in the cell, TcdC inhibits toxin transcription either by inhibiting the TcdR–core RNA polymerase interaction or by hampering the ability of the TcdR-containing RNA polymerase holoenzyme to recognize the toxin promoters (Matamouros *et al.*, 2007). In the absence of TcdC and in the presence of a positive stimulus, TcdR directs the core enzyme of the RNA polymerase to transcribe the toxin genes as well as its own encoding gene, resulting in a rapid increase in toxin production (Mani & Dupuy, 2001).

was initially thought to be the cause of the high toxin production phenotype of the epidemic strains (Warny *et al.*, 2005), but we have shown that a *tcdC* gene containing only the 18 bp deletion encodes a TcdC protein that is active both *in vivo* and *in vitro* (Matamouros *et al.*, 2007). Other *tcdC* alleles have also been reported (Spigaglia & Mastrantonio, 2002; MacCannell *et al.*, 2006) among the toxinogenic strains; recent molecular analysis of the epidemic strains showed that all of them carry not only in-frame deletions in the *tcdC* gene, but also a -1 frameshift mutation near the 5' end of the *tcdC* gene, resulting in a severely truncated form of TcdC protein (MacCannell *et al.*, 2006; Matamouros *et al.*, 2007).

The correlation between the high level of toxin production and the presence of a truncated form of TcdC in the epidemic strains is not yet fully understood. Warny and collaborators have demonstrated that toxin accumulates much faster and to a higher level in an epidemic strain than in a non-epidemic strain (Warny *et al.*, 2005). In this study, the first measure of toxin production was done at 24 h and toxinotype 0 strains were used as controls. In similar studies, we found that the absence of TcdC is most likely the cause of the accelerated kinetics of toxin production observed in the epidemic strains when compared to the non-epidemic toxinotype III strains (F. Barbut and others, unpublished data). Moreover, these preliminary data also showed that the non-epidemic toxinotype III strains produce higher toxin levels than other *C. difficile* toxinotype strains, although to a lesser extent than the epidemic strains. Thus the extreme cytotoxicity of the epidemic strain may not only be related to the disruption of the *tcdC* gene, but may also be due to intrinsic features of toxinotype III strains.

Other genetic characteristics that may contribute to the epidemic character of these strains may include the presence of the binary toxin genes and the acquisition of antibiotic-resistance determinants, which are important features for the dissemination and prevalence of *C. difficile* strains in hospitals.

Conclusions/summary

The correct timing of expression of virulence factors is generally crucial for the success of the organism as a pathogen and therefore expression of these factors has to be tightly regulated. In *C. difficile*, toxin gene expression is entirely dependent on the alternative σ factor TcdR, and toxin synthesis is clearly sensitive to several environmental factors. However, the molecular basis for the connection between toxin synthesis and exposure to specific environmental conditions is still unknown. In particular, it is not known whether toxin synthesis is regulated entirely through the modulation of TcdR synthesis and activity or whether it is regulated to some extent by direct control of the toxin genes. Recently, Dineen and collaborators have shown that CodY, a global regulator of gene expression in low G+C Gram-positive bacteria (Slack *et al.*, 1995),

represses the *tcdR* gene and, as a consequence, toxin gene expression in *C. difficile* (Dineen *et al.*, 2007). The intracellular signals to which CodY responds and the mechanism by which toxin gene expression is affected by the carbon source, temperature, biotin and various amino acids remain unknown. Therefore, it seems likely that several other regulatory factors will prove to be involved in the control of toxin gene expression and that the determination of their roles will unveil the full complexity of this regulation, thereby allowing us to better understand the pathogenesis of *C. difficile*.

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