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MicroReview

Regulation of toxin production in the pathogenic clostridia

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Summary

The genus *Clostridium* comprises a large, heterogeneous group of obligate anaerobic, Gram-positive spore forming bacilli. Members of this genus are ubiquitous in the environment and although most species are considered saprophytic, several are pathogenic to both humans and animals. These bacteria cause a variety of diseases including neuroparalysis, gas gangrene, necrotic enteritis, food poisoning, toxic shock syndrome and pseudomembraneous colitis, which in most cases arise as a consequence of the production of potent exotoxins. Treatment options are often limited, underscoring the need for new treatment strategies and novel therapeutics. Understanding the fundamental mechanisms and signals that control toxin production in the pathogenic clostridia may lead to the identification of novel therapeutic targets that can be exploited in the development of new antimicrobial agents.

Introduction

The pathogenic clostridia are an important group of bacteria that can cause disease in humans and other animals. Arguably the most important clostridial species in human disease are *Clostridium botulinum, Clostridium difficile, Clostridium perfringens, Clostridium sordellii* and *Clostridium tetani. C. botulinum* and *C. tetani* are neurotoxigenic and result in flaccid and spastic paralysis, respectively (Montecucco and Schiavo, 1994). *C. perfringens* is the causative agent of a myriad of different diseases ranging from gas gangrene and necrotic enteritis to food poisoning (Rood, 1998; Uzal and McClane, 2011), depending on the particular toxins produced by the infecting strain. *C. difficile* is an increasingly important nosocomial pathogen, which is considered the leading cause of antibiotic-associated diarrhoea and pseudomembranous colitis in the developed world (Carter *et al.*, 2011a; 2012) and *C. sordellii* is an emerging pathogen that causes a range of diseases including myonecrosis, sepsis and shock (Carter *et al.*, 2011a). The clostridial species that are important human pathogens also cause numerous neurotoxic, histotoxic and enterotoxic animal diseases. In addition to these species, *Clostridium chauvoei*, *Clostridium colinum*, *Clostridium novyi*, *Clostridium septicum* and *Clostridium spiroforme* also cause severe disease in numerous domestic and livestock animals (Songer, 1996; 1998; 2010). Although these organisms are responsible for very different diseases, in most cases the symptoms of disease are associated with the production of potent exotoxins that result in damage to the host (Stevens *et al.*, 2012). Collectively the pathogenic clostridia produce some of the most potent toxins known, including the *C. botulinum* neurotoxin (BoNT), which is the most potent toxin identified (Schechter and Arnon, 2000; Katona, 2012).

Many of the clostridial toxins have been purified and subjected to extensive structural and functional analysis. However, understanding the mechanisms and signals that control their production has been limited by the difficulties encountered in genetically manipulating the pathogenic clostridia. Indeed, the construction of genetically defined mutants in many clostridial species remains a challenge and methods have yet to be developed for successful genetic manipulation of these bacteria. However, the recent development of techniques that have facilitated the genetic manipulation of some of these species (Lyristis *et al.*, 1994; Chen *et al.*, 2005; O'Connor *et al.*, 2006; Heap *et al.*, 2007; Bradshaw *et al.*, 2010; Carter *et al.*, 2011a) has resulted in a deeper understanding of the genes and regulatory pathways that control toxin production at the molecular level. One important outcome of this recent work may be that novel targets will be identified and targeted in the development of therapeutics for the treatment of these devastating clostridial infections, for which few effective remedies are currently available. Here, we summarize and discuss the current understanding of the regulatory mechanisms and signals used to control toxin production

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in the pathogenic clostridia, with a particular emphasis on *C. botulinum, C. difficile, C. perfringens, C. sordellii* and *C. tetani*.

Nutritional and environmental stimuli

Environmental stimuli play an important role in controlling toxin production in the pathogenic clostridia. For example, the production of α-toxin, β-toxin, β2-toxin and perfringolysin O (PFO) is activated following direct cell–cell contact with eukaryotic host cells such as Caco-2 cells (Vidal *et al*., 2009b). Temperature is also able to modulate toxin production in the clostridia, with the production of α-toxin in *C. perfringens* being optimal at 25°C (Katayama *et al*., 1999), whereas in *C. difficile* maximal toxin production occurs at 37°C, with reduced yields at 22°C and 42°C (Karlsson *et al*., 2003). A number of other environmental stimuli also have an impact on toxin production in *C. difficile*, including bicarbonate concentration (Karlsson *et al*., 1999), sub-inhibitory concentrations of certain antibiotics (Chilton *et al*., 2012; Aldape *et al*., 2013) and the presence of short-chain fatty acids such as butyric acid (Karlsson *et al*., 2000), although the molecular mechanisms controlling these responses are not well understood. One of the most important groups of environmental cues is likely to be nutritional signals. In *C. botulinum,* the presence of arginine and derivatives such as proline, glutamate and ammonia repress neurotoxin production (Johnson and Bradshaw, 2001), as does the aromatic amino acid tryptophan (Johnson and Bradshaw, 2001). Similarly, amino acids such as proline, cysteine, and certain branched chain amino acids (BCAAs), repress toxin production in *C. difficile* (Karlsson *et al.,* 1999). In this bacterium, the global transcriptional regulator CodY appears to be responsible for mediating the observed amino acid-based repression, with increased amounts of toxin produced by a *C. difficile codY* mutant in the presence of BCAAs compared to the wild-type strain (Dineen *et al*., 2007). CodY represses toxin production predominately by repressing the expression of *tcdR*, a critical regulator of toxin production, which is discussed in more detail later in this review. In this instance, the presence of BCAAs results in increased CodY binding to the *tcdR* promoter and the subsequent repression of *tcdR* expression (Dineen *et al*., 2010), leading to reduced toxin production.

Catabolite repression

The presence of glucose, or other rapidly metabolizable carbon sources, is also an important nutritional signal that inhibits the production of several toxins in the pathogenic clostridia, including toxin A (TcdA) and toxin B (TcdB) in *C. difficile* (Dupuy and Sonenshein, 1998), and α-toxin and PFO in *C. perfringens* (Mendez *et al*., 2012). In both organisms, carbon catabolite repression is thought to be responsible since glucose inhibition of toxin production is not observed in *ccpA* mutants that no longer produce the carbon catabolite repressor protein CcpA (Antunes *et al*., 2011; Mendez *et al*., 2012). Further support for the role of CcpA in mediating glucose-dependent inhibition of toxin production in *C. difficile* comes from the finding that the *tcdA*, *tcdB* and toxin regulator genes *tcdR* and *tcdC* are direct targets of the CcpA protein *in vitro* (Antunes *et al*., 2012). The observation that *C. difficile ccpA* mutants produce a reduced amount of toxin in comparison to the wild-type strain (Antunes *et al*., 2011) suggests that toxin regulation is complex and that unidentified regulators must also be involved in the CcpA-regulation cascade. In *C. perfringens*, CcpA also regulates *C. perfringens* enterotoxin (CPE) production in a growth phase-dependent manner that is not linked to the presence of glucose (Varga *et al*., 2004), suggesting that unknown factors must play a role in CcpA-mediated regulation of CPE.

Growth phase and quorum sensing

Growth phase signals also play an important role in the regulation of toxins by the pathogenic clostridia. The production of α-toxin (Vidal *et al*., 2009b), PFO (Vidal *et al*., 2009b), NetB (Cheung *et al*., 2010), β-toxin (Fernandez-Miyakawa *et al*., 2007) and ε-toxin (Fernandez-Miyakawa *et al*., 2007) by *C. perfringens* occurs during or after late exponential growth, while CPE is produced exclusively during sporulation (Duncan, 1973). Unlike most clostridial toxins, CPE is not actively secreted; instead, it accumulates in the mother cell during sporulation before being released along with the spore when the mother cell lyses (Duncan, 1973). In *C. botulinum* and *C. tetani*, the production of BoNT and TeNT, respectively, is associated with the transition to stationary-phase growth (Mellanby and Green, 1981; Couesnon *et al*., 2006), as is the production of TcdA and TcdB in *C. difficile* (Hundsberger *et al*., 1997). The precise growth phase signals involved in toxin production remain mostly unknown; however, the role of quorum sensing has become increasingly clear in recent years.

Accessory gene regulator (*agr*)-based quorum sensing

The accessory gene regulator (*agr*) system of *S. aureus*, the most extensively characterized quorum sensing system of any Gram-positive pathogen, regulates the expression of many virulence factors in this bacterium, including several toxins (Novick, 2003). This system comprises the *agrAC* genes, encoding a two-component signalling system, and the *agrBD* genes, which encode an autoinducing peptide (AIP) processing and export protein and the AIP pre-peptide, respectively (Novick, 2003). Homologues of the *S. aureus agrBD* genes have been

	Amphipathic Region	AIP	Charged Region	
Sau-group I	MNTLFNLFFDFITGILKNIGNIAA	YSTCDFIM	DEVEVPKELTQLHE	46
Sau-group II	MNTLVNMFFDFIIKLAKAIGIVG	GVNACSSLF	DEPKVPAELTNLYDK	47
Sau-group III	MKKLLNKVIELLVDFFNSIGYRAAY	INCDFLL	DEAEVPKELTQLHE	46
Sau-group IV	MNTLYKSFFDFITGVLKNIGNVAS	YSTCYFIM	DEVEIPKELTQLHE	46
Cpe-CN3685 AgrD	MKKLNKNLLTLFAALTTVVATTVA	TSACLWFT	HQPEEPKSLRDE	44
Cbo-3502 AgrD1	MKKLNKKVLMLVATFTTLLASIVA	SSACYWCV	YQPKEPKCLREE	44
Cbo-3502 AgrD2	MKKQLKEKCTKVTAKLLKSVAYSTA	DSACHLGI	YQPKEPKSLRK	44
Cdi-R20291 AgrD1	MKKFIVRFMKFASSLALSTAILSA	NSTCPWII	HQPKVPKEISNLKKTN	48
Cdi-R20291 AgrD2	MKKIALNLLKNISALSFGIAVLSA	NSASSWVA	HQAKEPQALQKLKK	46
Cno-19402 AgrD	MKIMNKLSKGIAKTISKISTDVAYTSTE	ACVSLNGL	EEPKMPKVLLKKTK	50
Cso-9714 AgrD1	MNFLTNLFSDFALALGNGA	STMCSFIF	FEPEMPKSKRDN	39
Cso-9714 AgrD2	MDFKKKIGSLMALLTVFTVGA	GAASMLAV	GVEDMPKSLKEKR	42
Cso-9714 AgrD3	MDFKKIIGDLMASLTLLTAGA	GTASMLAV	GVEDMPKSLKEKR	42
Cch-JF4335 AgrD	MRKLNNKVLMAVAAFATVFASVVA	TSACVWCS	YQPEEPKCLRDK	44
Cse-BX96 AgrD	MRKLNKKVLVGVATFATVFASVIA	TSACFWCT	YQPKEPKCLRDK	44

Fig. 1. Alignment of confirmed and putative AgrD amino acid sequences. The alignment shows the empirically confirmed AgrD peptides from *S. aureus* groups I–IV (*Sau*-group I–IV) (Otto, 2001), *C. perfringens* strain CN3685 (*Cpe*-CN3685 AgrD) (Vidal *et al*., 2012) and *C. botulinum* strain ATCC3502 (*Cbo*-3502 AgrD1/AgrD2) (Cooksley *et al*., 2010). Putative AgrD peptides identified by bioinformatic analysis of the genome sequences of *C. difficile* strain R20291 (*Cdi*-R20291 AgrD1/AgrD2), *C. novyi* strain ATCC19402 (*Cno*-19402 AgrD), *C. sordellii* strain ATCC9714 (*Cso*-9714 AgrD1/AgrD2/AgrD3), *C. chauvoei* strain JF4335 (*Cch*-JF4335 AgrD) and *C. septicum* strain BX96 (*Cse*-BX96 AgrD) are also shown. The peptides are split into three regions representing the N-terminal amphipathic region, the AIP encoding region and the C-terminal charged region. The amino acids predicted or confirmed to form the thiolactone/lactone ring structure of the mature AIP are highlighted in yellow and a highly conserved proline residue found in all confirmed and putative AgrD peptides is highlighted in blue.

identified in a number of the toxigenic clostridia, including *C. difficile* (Sebaihia *et al*., 2006), *C. botulinum* (Cooksley *et al*., 2010) and *C. perfringens* (Ohtani *et al*., 2009, Vidal *et al*., 2009a) (Fig. 1). It is evident that *agr*-based quorum sensing plays an important role in controlling toxin production in both *C. botulinum* and *C. perfringens*. *C. botulinum* contains two homologues of the *agrBD* genes, named *agrBD1* and *agrBD2* (Cooksley *et al*., 2010). Inactivation of either *agrD1* or *agrD2* resulted in lower BoNT production compared to the wild-type strain, with the latter mutant displaying the greatest reduction (Cooksley *et al*., 2010). Similarly, inactivation of the *agrBD* genes has a profound effect on toxin production in *C. perfringens*. Mutants in *agrB* in a number of different strain backgrounds display a complete loss of PFO production (Ohtani *et al*., 2009; Vidal *et al*., 2009a), and reduced levels of α-toxin (Ohtani *et al*., 2009; Vidal *et al*., 2009a), κ-toxin (Ohtani *et al*., 2009), CPE (Li *et al*., 2011), β-toxin (Vidal *et al*., 2012), β2-toxin (Li *et al*., 2011) and ε-toxin (Chen *et al*., 2011a) production compared to the respective wild-type strains. Importantly, toxin production in a *C. perfringens agrB* mutant could be restored upon the addition of a synthetic *C. perfringens* AgrD peptide to an *in vitro* grown culture, supporting the hypothesis that *agr*-based quorum sensing controls toxin production in this bacterium (Vidal *et al*., 2012). Further evidence of the role of the *C. perfringens agr* system in modulating virulence was obtained using both a rabbit small intestinal loop model and a mouse challenge model, which confirmed that the *agrB* mutant was attenuated in virulence (Vidal *et al*., 2012). The situation is less clear in *C. difficile* since no *agrBD* mutants have been reported. However, a recent study showed that insertional inactivation of *agrA*, a response regulator located in the *agr2* locus, in strain R20291 resulted in a twofold reduction in *tcdA* gene expression and a colonization defect in a mouse model of CDI. These results suggest that the *agr2* quorum sensing system might play a role in regulating the virulence response of this organism (Martin *et al*., 2013). Since similar *agrBD* homologues appear widespread among the clostridia, it is likely that future research will show that similar quorum-sensing systems play an important role in controlling virulence factor expression in many toxigenic clostridial species, which may provide important targets for therapeutic development.

Two-component signal transduction systems

The most common way by which bacteria detect and respond to changes in their environment is through two-component signal transduction systems. These phospho-relay systems generally consist of a sensor histidine

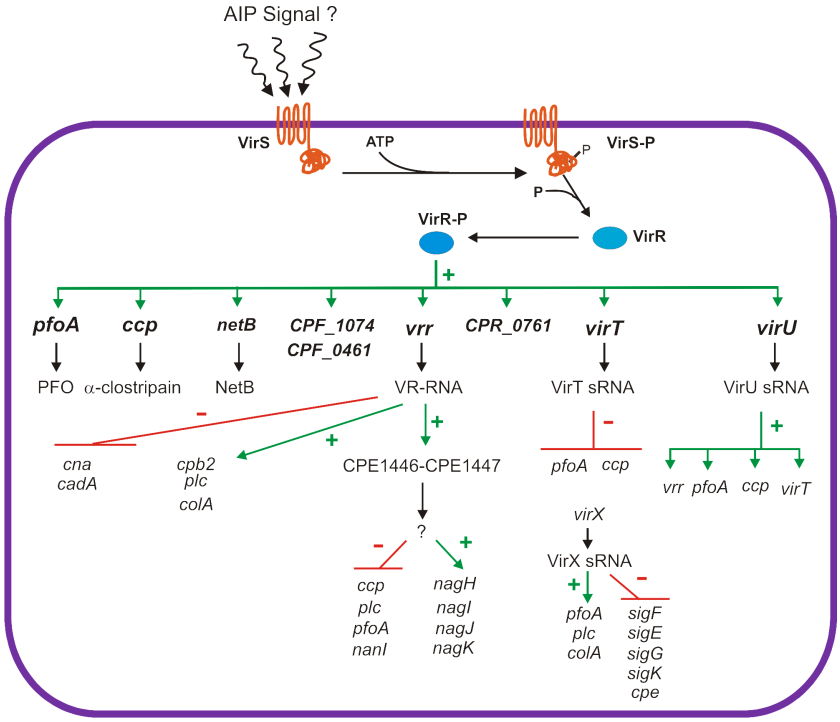

Fig. 2. Model of the regulation of gene expression by the VirSR two-component signal transduction system. Positive regulation is indicated by the green arrow and plus symbols. Negative regulation is denoted by red lines and minus symbols. Upon detection of an external signal (potentially an AIP), the VirS sensor histidine kinase (shown in orange) autophosphorylates and then becomes the phospho-donor for its cognate response regulator, VirR (shown in blue). Phosphorylated VirR directly regulates the expression of *pfoA*, *ccp*, *netB*, *vrr*, *virT*, *virU*, *CPF_1074*, *CPF_0461* and *CPR_0761* by binding to the VirR boxes located in the promoter regions located upstream of these genes. VirR indirectly regulates the indicated toxin or virulence factor genes via the VR-RNA, VirU or VirT regulatory RNA molecules. The VR-RNA-controlled CPE1446/CPE1447 system positively regulates the expression of hyaluronidase genes but negatively regulates *ccp*, *plc*, *pfoA* and *nanI* transcription. The VirSR-independent VirX sRNA positively regulates the expression of *pfoA*, *plc* and *colA*, and negatively controls the transcription of *cpe* and the sporulation sigma factor genes *sigF*, *sigE*, *sigG* and *sigK*. Adapted from Cheung *et al.* (2013).

kinase that detects a specific environmental signal, and a response regulator that converts that signal into a cellular response by controlling the expression of a particular subset of genes (Capra and Laub, 2012).

Among the pathogenic clostridia, the best-studied two-component system is found in *C. perfringens*. The production of several toxins, as well as many extracellular enzymes, is regulated by the VirSR two-component signal transduction system, with inactivation of either *virS* or *virR* resulting in an altered toxin production profile compared to the wild-type strain (Lyristis *et al.*, 1994; Shimizu *et al.*, 1994; Ohtani *et al.*, 2003; Cheung *et al.*, 2010; Ma *et al.*, 2011) and in the case of *virS*, an attenuated *in vivo* virulence phenotype in a mouse myonecrosis model (Lyristis *et al.*, 1994). This global regulatory network consists of the VirS sensor histidine kinase and its cognate response regulator, VirR (Lyristis *et al.*, 1994; Shimizu *et al.*, 1994). The regulatory cascade (Fig. 2) begins with the detection of a specific signal by VirS. For many years the nature of this signal was unknown, but recent work has suggested that one signal might be the AgrD quorum sensing AIP (Ohtani *et al.*, 2009), described earlier. Following the detection of a stimulus, VirS autophosphorylates and then donates the phosphoryl group to VirR, leading to its activation. The activated VirR is then able to control the transcription of its target genes (Cheung *et al.*, 2009).

The genes comprising the VirSR regulon can be divided into two groups. The first group consists of six genes that are directly activated by VirR (Cheung *et al.*, 2010; Ohtani *et al.*, 2010), and includes the toxin genes *pfoA* (Cheung *et al.*, 2004) and *netB* (Cheung *et al.*, 2010), which encode for PFO and NetB toxin respectively. The second group in *C. perfringens* strain 13, includes 147 genes that VirR activates indirectly (Ohtani *et al.*, 2010), again including several toxin genes such as *plc*, *colA* (Banu *et al.*, 2000; Shimizu *et al.*, 2002) and *cpb2* (Ohtani *et al.*, 2003), which encode α-toxin, κ-toxin and β2-toxin, respectively. Direct regulation of genes is mediated through binding of VirR to regions known as VirR boxes in the promoter regions of target genes (Cheung *et al.*, 2004). VirR boxes contain two imperfect direct repeats that are essential for VirR-regulated transcription (Cheung and Rood, 2000). The maintenance of correct helical phasing and spacing between the VirR boxes and the –35 region of target gene promoters is also critical for optimal transcriptional activation (Cheung *et al.*, 2004). Indirect regulation involves a different mechanism that utilizes an intermediate regulatory RNA molecule known as VR-RNA (Shimizu *et al.*, 2002), discussed later in this review, the expression of which is positively controlled by VirR (Shimizu *et al.*, 2002). It is noteworthy that *virSR* gene homologues have been identified in a number of the pathogenic clostridia; however, the role of these genes in regulating toxin production is yet to be determined.

Two-component signal transduction systems are also important in controlling the production of BoNT in *C. botulinum*. Antisense RNA targeted against three two-component systems, CLC_1093/CLC_1094, CLC_1914/CLC_1913 and CLC_0661/CLC_0663, resulted in the transcriptional activation of genes encoding both BoNT

and the associated non-toxic protein (ANTP) (Connan *et al*., 2012), suggesting that these systems positively regulate neurotoxin production. Whether this regulation is direct or indirect, or if there is cross-talk between the three systems, remains to be determined. By contrast, the CBO0786/CBO0787 two-component system of *C. botulinum* strain ATCC3502 was found to negatively regulate BoNT production (Zhang *et al*., 2013), with mutants in either the gene encoding the sensor histidine kinase (*cbo0787*) or the response regulator (*cbo0786*) found to produce significantly higher levels of BoNT than the wild-type strain (Zhang *et al*., 2013). Experiments performed *in vitro* demonstrated that the CBO0786 response regulator bound to the BoNT and ANTP promoter regions, suggesting a direct mechanism of toxin inhibition (Zhang *et al*., 2013). The CBO0786 DNA binding sites identified by DNase I footprinting do not, however, appear to contain any repeat sequences, a characteristic feature of most other response regulator binding sites, and appear to show similarity only in the –10 box of the target gene promoters. This unexpected finding suggests that CBO0786 must use an as yet unknown mechanism to distinguish these binding sites from the promoters of other genes with similar pribnow boxes.

In *C. difficile*, toxin production is also influenced by several two-component signalling systems. For example, CDT-binary toxin is positively regulated by an orphan response regulator known as CdtR, which is located immediately upstream of the *cdtA* gene and within the CdtLoc, a chromosomal region encoding the structural binary toxin genes, *cdtA* and *cdtB*, as well as *cdtR* (Carter *et al*., 2007). In addition, Spo0A, the master regulator of sporulation in both *Bacillus* and *Clostridium* species, may play a role in regulating TcdA and TcdB production. Note that in *C. perfringens* Spo0A has previously been shown to regulate the production of CPE (Huang *et al*., 2004) and TpeL (Paredes-Sabja *et al*., 2011), providing evidence that this regulator plays a role in both clostridial toxin production and sporulation in this bacterium. At this time, however, the exact role of Spo0A in *C. difficile* is unclear, with a number of studies presenting conflicting data. In one of these studies, Spo0A positively regulated toxin production in strain 630Δ*erm* (Underwood *et al*., 2009), while in another study Spo0A appeared to play no role in toxin production in the same strain (Rosenbusch *et al*., 2012). A third study using strain R20291, an epidemic BI/NAP1/027 isolate, showed that Spo0A negatively regulated toxin production (Deakin *et al*., 2012). The apparent discrepancy between these studies was clarified by a recent publication that suggests that Spo0A exerts differential regulatory effects on toxin production in different *C. difficile* strain backgrounds. In this work, the construction and complementation of *spo0A* mutants in two ribotype 027 isolates, M7404 and R20291, demonstrates that Spo0A acts as a negative regulator of TcdA and TcdB production in this strain background while equivalent mutations in 630Δ*erm* and a ribotype 078 isolate, JGS6133, did not alter toxin production (Mackin *et al*., 2013). These results suggest that Spo0A regulation of toxin production in *C. difficile* may be an adaptation specific to ribotype 027 isolates.

Alternative sigma factors

In addition to two-component signalling systems, multiple alternative sigma factors regulate toxin production in the pathogenic clostridia. One such example is Sigma H (SigH), which is involved in the transition to stationary-phase growth and sporulation in both *Bacillus* and *Clostridium* species (Sauer *et al*., 1994). SigH negatively regulates toxin production in *C. difficile,* with a *sigH* mutant found to produce increased amounts of TcdA and TcdB in comparison to the wild-type strain (Saujet *et al*., 2011). By contrast, CPE production in *C. perfringens* is positively regulated by alternative sigma factors, with significantly reduced or abolished CPE expression observed in *sigE, sigF* and *sigK* null mutants that no longer produce the sporulation sigma factors Sigma E (SigE), Sigma F (SigF) and Sigma K (SigK) (Harry *et al*., 2009; Li and McClane, 2010). Three promoters, P1, P2 and P3, are responsible for coupling CPE production with sporulation (Zhao and Melville, 1998). Promoter P1 is similar to consensus SigK promoters while promoters P2 and P3 show similarity to other SigE-dependent promoters. The regulatory cascade controlling CPE production appears to be hierarchical since SigF regulates the expression of *sigE* and *sigK*, which then directly control *cpe* transcription from the SigE and SigK-dependent *cpe* promoters (Li and McClane, 2010).

The TcdR family of alternative sigma factors

The most thoroughly studied alternative sigma factors in the clostridia are the TcdR family of proteins. These proteins are members of the extracytoplasmic function (ECF) family of alternative sigma factors, which belong to group 5 of the σ^{70} family (Mani and Dupuy, 2001). TcdR is the prototype member of this family and is critical for the initiation of toxin production in *C. difficile* (Mani and Dupuy, 2001; Mani *et al*., 2002). TcdR homologues have recently been identified in species other than *C. difficile* (Fig. 3) and in some but not all cases are involved in the regulation of toxin production (Dupuy *et al*., 2006). BotR and TetR, for example, control the expression of BoNT and TeNT by *C. botulinum* and *C. tetani,* respectively (Raffestin *et al*., 2005), while TcsR controls the production of TcsL and TscH in *C. sordellii* (Sirigi Reddy *et al*., 2013). These proteins are similar enough to substitute for one another in DNA binding

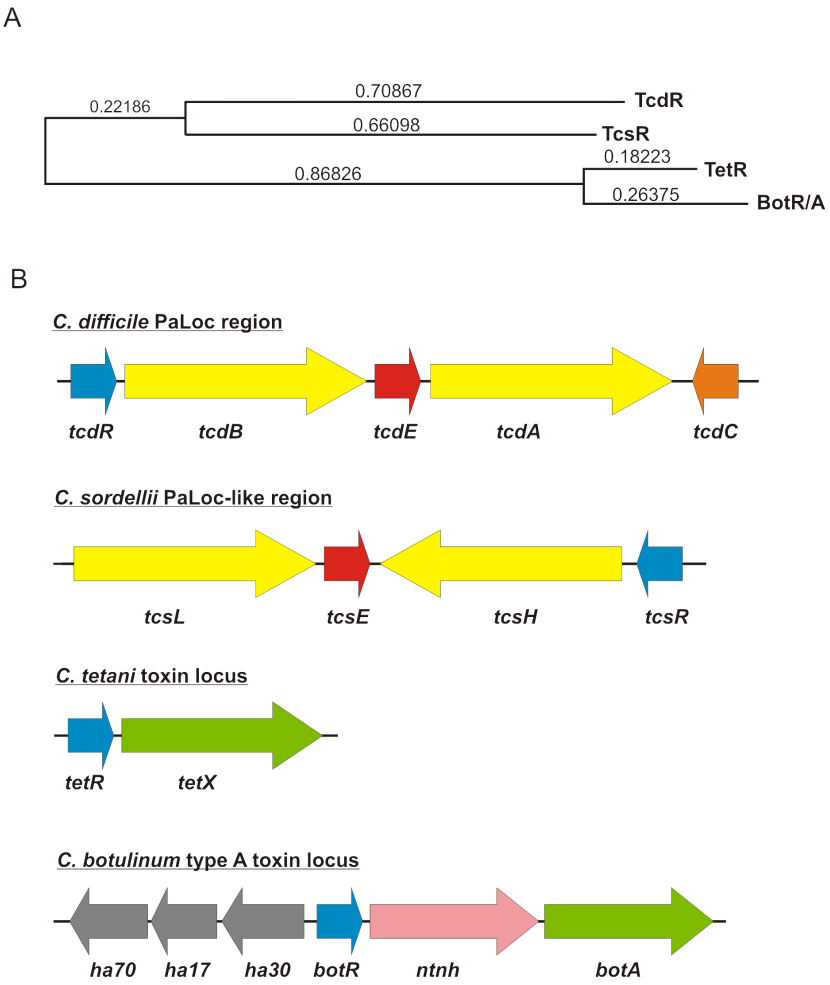

Fig. 3. A. Phylogenetic tree showing the genetic relatedness of toxin-associated TcdR family proteins at the amino acid level. Distance values represent the divergence of each protein as the number of amino acid substitutions in proportion to the total length of the protein alignment. Multiple sequence alignment was performed using the CLUSTALW algorithm available at: http://www.ebi.ac.uk/tools/msa/clustalw2. Phylogenetic analysis of the TcdR multiple sequence alignment was performed using the neighbour-joining method available at: http://www.ebi.ac.uk/tools/phylogeny/clustalw2_phylogeny.
B. Schematic representation of toxin loci and genes regulated by TcdR family proteins in the pathogenic clostridia. The PaLoc region of *C. difficile* (Braun *et al.*, 1996) is shown, which harbours the toxin structural genes *tcdA* and *tcdB,* and the accessory genes *tcdR, tcdE* and *tcdC*. The recently identified PaLoc-like region of *C. sordellii* (Sirigi Reddy *et al.*, 2013) encoding the toxin genes *tcsL* and *tcsH* and the accessory genes *tcsR* and *tcsE* is also shown. The *C. tetani* locus (Dupuy *et al.*, 2006) contains the TeNT toxin gene *tetX* and the accessory gene *tetR* while the *C. botulinum* type A toxin locus (Dupuy *et al.*, 2006) contains the BoNT toxin gene *botA* and the accessory gene *botR*. In addition to these genes, the *C. botulinum* locus encodes the non-toxic-non-haemagglutinin (*ntnh*) gene and the haemagglutinin genes (*ha70, ha17* and *ha30*), the products of which form part of the BoNT complex. Similarly coloured genes encode for functionally related proteins: blue, TcdR family alternative sigma factors; yellow, large clostridial toxins; green, neurotoxins; red, putative holin-like proteins; orange, anti-sigma factor; pink, non-toxic-non-haemagglutinin protein and grey, haemagglutinin proteins.

assays and run-off transcription experiments *in vitro* (Dupuy *et al.*, 2006). However, only BotR and TetR (Dupuy *et al.*, 2006) or TcdR and TcsR (Sirigi Reddy *et al.*, 2013), respectively, were functionally interchangeable *in vivo*. This is in keeping with the phylogenetic relatedness of *C. difficile* and *C. sordellii*, which were recently shown to be members of the Peptostreptococcaceae family and not the Clostridiaceae family to which *C. botulinum* and *C. tetani* belong, although both families belong to the order Clostridiales (Yutin and Galperin, 2013). The variations in functional interchangeability possibly reflect the divergence within regions 2.4 and 4.2 of these proteins, which mediate binding to the –10 and –35 regions of their target toxin gene promoters, respectively (Dupuy *et al.*, 2006). Note that while the –35 region of the target genes is highly conserved, the –10 region is more variable (Dupuy *et al.*, 2006) which may consequently restrict promoter recognition by TcdR family proteins in more distantly related species. Nevertheless, given the overall level of similarity between these proteins, it seems likely that they arose from a common ancestor and subsequently co-evolved to develop a degree of specificity with the toxin genes that they regulate.

The pathogenicity locus (PaLoc)

In *C. difficile*, the *tcdA* and *tcdB* toxin genes and the *tcdR* gene are located within a 19.6 kb chromosomal pathogenicity locus, known as PaLoc (Braun *et al.*, 1996) (Fig. 3B). This region contains two additional genes, *tcdE,* which appears to encode a bacteriophage holin-like protein possibly involved in toxin export (Govind and Dupuy, 2012), and *tcdC,* a putative anti-sigma factor (Carter *et al.*, 2011b). The *tcsL* and *tcsH* toxin genes of *C. sordellii* were also shown to reside within a PaLoc-like region (Fig. 3B) that also harbours *tcsR* and a gene with homology to the *C. difficile tcdE* gene (Sirigi Reddy *et al.*, 2013). Note that TcsL and TcsH belong to the large clostridial toxin, or LCT, family, which also includes *C. difficile* TcdA (Sullivan *et al.*, 1982) and TcdB (Sullivan

et al., 1982), TcnA from *C. novyi* (Bette *et al*., 1991) and TpeL from *C. perfringens* (Amimoto *et al*., 2007). The observation that *C. sordellii tcsL* and *tcsH* are found in a PaLoc region similar to that found in *C. difficile* suggests that the LCT genes might all be located within similar loci. Since *C. difficile* PaLoc appears to be of bacteriophage origin (Braun *et al*., 1996) and *tcnA* and *tpeL* from *C. novyi* and *C. perfringens* are associated with a lysogenic bacteriophage and a conjugative plasmid, respectively, the movement of these toxin loci between species from a common ancestor seems highly plausible. Whether *tcnA* and *tpeL* also reside within PaLoc-like regions is currently unknown.

TcdC

The TcdC protein is thought to be a negative regulator of toxin production in *C. difficile* (Dupuy *et al*., 2008). Interest in this regulator increased when it was reported that epidemic BI/NAP1/027 strains carry a nonsense mutation within *tcdC* (Warny *et al*., 2005), leading to the hypothesis that TcdC inactivation may be responsible for the increased virulence of these strains (Warny *et al*., 2005). Despite numerous studies designed to address the role of TcdC in toxin production and virulence, the picture remains unclear, with conflicting findings reported (Matamouros *et al*., 2007; Carter *et al*., 2011b; Bakker *et al*., 2012; Cartman *et al*., 2012). In one study, TcdR-dependent *in vitro* transcription assays in the presence of purified TcdC protein, as well as assays performed in the surrogate host *C. perfringens* using a *tcdA* promoter-reporter gene fusion, showed that TcdC represses toxin gene expression by interfering with binding of TcdR-associated RNA polymerase to the PaLoc gene promoters (Matamouros *et al*., 2007). However, a second study showed that the insertional inactivation of the *tcdC* gene in *C. difficile* strain 630Δ*erm* had little impact on toxin production (Bakker *et al*., 2012). In a third study, the introduction of a plasmid-borne copy of *tcdC* into the BI/NAP1/027 strain M7404 resulted in the downregulation of toxin production and an attenuated virulence phenotype in the hamster model of infection (Carter *et al*., 2011b). However, in a fourth study, correction of the *tcdC* mutation on the chromosome of the BI/NAP1/027 isolate R20291 resulted in no discernible effect on toxin production (Cartman *et al*., 2012). The reasons for these conflicting data are not clear, but may be related to experimental variation between the studies, such as different growth media and strains being used, or it might be that other important differences account for the disparate *in vitro* and *in vivo* results. Whatever the reasons, further work is needed to conclusively define the role of TcdC in *C. difficile*, particularly on virulence capacity. However, based on currently available data it is unlikely that the *tcdC* mutation alone is responsible for the increased virulence of BI/NAP1/027 isolates (Carter *et al*., 2011b).

Small regulatory RNAs

In addition to protein regulators, bacteria utilize another class of regulatory molecule known as small regulatory RNAs (sRNA). These RNA regulators can vary in length from 50 to 300 nucleotides and act either *in cis* or *in trans* (Storz *et al*., 2011). The majority of sRNAs interact with mRNA targets through an antisense mechanism and can alter transcription, translation and/or mRNA stability of target genes (Lalaouna *et al*., 2013). By use of *in silico* sRNA prediction algorithms and deep sequencing, putative sRNAs have been identified in several clostridial genomes including *C. difficile* (Soutourina *et al*., 2013), *C. botulinum* (Chen *et al*., 2011b), *C. tetani* (Chen *et al*., 2011b), *C. novyi* (Chen *et al*., 2011b) and *C. perfringens* (Chen *et al*., 2011b). With the exception of *C. perfringens*, none have been shown to regulate toxin production, although it seems likely that sRNA-mediated regulation of toxin production in the pathogenic clostridia will be experimentally proven in the future, as has been found in other bacteria.

In *C. perfringens* four sRNA molecules, known as VR-RNA, VirU, VirT and VirX, have been shown to regulate the production of various toxins. Of these, the best characterized is VR-RNA. This regulatory RNA is encoded by the *vrr* gene and is the crucial link between the VirSR system and the genes targeted in an indirect manner by this regulatory system. The mechanism by which VR-RNA regulates target gene expression is not well understood but appears to be dependent on the specific target mRNA. Regulation of κ-toxin expression, for example, relies on base pairing of the 3′ region of VR-RNA with the 5′ untranslated region (UTR) of the κ-toxin *colA* gene mRNA. This results in the disruption of a stem loop structure within the 5′ UTR and the exposure of a sequestered ribosome binding site. This conformational change also leads to cleavage of the *colA* mRNA, resulting in stabilization of the transcript and increased levels of translation (Obana *et al*., 2010). Although the 3′ end of VR-RNA was also found to be important for α-toxin expression, there does not seem to be any complementarity with this region and the 5′ UTR of *plc* mRNA, suggesting that a different mechanism to that described for κ-toxin is utilized.

The second sRNA, called VirU, has been shown to increase the levels of *vrr*, *pfoA* and *virT* mRNA, suggesting that it acts as a positive regulator or that it increases the stability of these mRNAs (Okumura *et al*., 2008). By contrast, VirT was found to negatively regulate the transcription of *pfoA* and *colA* (Okumura *et al*., 2008). The mechanism by which these sRNAs control their targets remains unclear, but it is postulated that they might be

involved in preserving balanced gene expression by fine-tuning the transcription of VirSR-regulated genes (Okumura *et al*., 2008).

The final sRNA, known as VirX, activates transcription of the *plc, colA* and *pfoA* genes (Ohtani *et al*., 2002), and suppresses expression of CPE (Ohtani *et al*., 2013). While the exact mechanism by which VirX regulates *plc, colA* and *pfoA* gene expression remains to be determined, the control of CPE production is thought to result from VirX-mediated downregulation of the sporulation sigma factors SigF, SigE and SigK, which are important for activation of CPE production during sporulation (Ohtani *et al*., 2013), as discussed earlier.

Conclusions and future perspectives

The development of new technologies that have facilitated the study of the pathogenic clostridia at the molecular level have, for the first time, allowed the regulatory cascades and environmental signals that control the production of toxins in these bacteria to be dissected. The regulatory pathways involved are complex and multi-faceted, and detailed research is needed before a comprehensive understanding is achieved. The use of *in vivo* experiments is of particular importance, since in many cases it is not clear whether *in vitro* conditions used to study toxin production are truly reflective of the *in vivo* conditions that induce the production of these toxins. Nevertheless, it is becoming increasingly clear that toxin production between different clostridial species involves conserved regulatory mechanisms including quorum sensing, alternative sigma factors and two-component signalling systems. Importantly, with the development of new antibiotics in decline, understanding these regulatory networks may identify important new targets for the development of novel antimicrobial or anti-virulence compounds that act against the pathogenic clostridia and that may be efficacious in treating diseases caused by this group of bacteria.

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