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Expression of the large clostridial toxins is controlled by conserved regulatory mechanisms

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ABSTRACT

The clostridia cause many human and animal diseases, resulting in significant morbidity and mortality. Host damage results from the action of potent exotoxins, an important group of which is the large clostridial toxins (LCTs) produced by *Clostridium difficile*, *Clostridium sordellii*, *Clostridium perfringens* and *Clostridium novyi*. Knowledge of the structure and function of these toxins has been attained, however, apart from *C. difficile*, the regulatory pathways that control LCT production remain largely unknown. Here we show that LCT production in *C. sordellii* and *C. perfringens* is temporally regulated and repressed by glucose in a similar manner to *C. difficile*. Furthermore, we show that the TpeL-encoding gene of *C. perfringens* is located in an uncharacterized Pathogenicity Locus (PaLoc), along with accessory genes predicted to encode a bacteriophage holin-type protein and a TcdR-family alternative sigma factor, TpeR. Inactivation of *tpeR* demonstrated that TpeR is critical for *C. perfringens* TpeL production, in a similar manner to *C. difficile* TcdR and *C. sordellii* TcsR, but cross-complementation showed that TpeR is not functionally interchangeable with TcdR or TcsR. Although conserved mechanisms are employed by the clostridia to control LCT production there are important functional differences that distinguish members of the TcdR-family of clostridial alternative sigma factors.

Introduction

The large clostridial toxin (LCT) family includes toxin A (TcdA) and toxin B (TcdB) from *Clostridium difficile* (Jank and Aktories, 2008), lethal toxin (TcsL) and haemorrhagic toxin (TcsH) from *Clostridium sordellii* (Voth et al., 2006), alpha toxin (TcnA) from *Clostridium novyi* (Busch et al., 2000) and TpeL from *Clostridium perfringens* (Amimoto et al., 2007). These toxins are all monoglycosyltransferases that inactivate Rho family GTPases through the covalent transfer of a glucose or *N*-acetylglucosamine moiety. Cellular intoxication with a LCT results in disruption of the actin cytoskeleton, cell rounding and, eventually, apoptosis and cell death (Jank and Aktories, 2008; Voth et al., 2006). The importance of the LCTs in disease is becoming increasingly clear, and there is now mounting evidence to suggest that some of these toxins are essential virulence factors (Carter et al., 2011a; Dang et al., 2001; Lyras et al., 2009). Furthermore, the LCTproducing clostridia are important human and animal pathogens that cause severe disease and are increasingly being associated with high rates of morbidity and mortality (Aldape et al., 2006; Majumdar et al., 2004; Redelings et al., 2007).

With the exception of TcdA and TcdB from *C. difficile*, very little is known about how the clostridia regulate the expression of the LCTs. In *C. difficile*, TcdA and TcdB are encoded by *tcdA* and *tcdB*, respectively, within a chromosomal region known as the Pathogenicity Locus or PaLoc. In addition to the toxin genes, three accessory genes are encoded within PaLoc, designated *tcdR*, *tcdE* and *tcdC*. Substantial experimental evidence suggests that *tcdR* encodes an alternative sigma factor, TcdR, which is critical for the expression of both toxins A and B (Mani and Dupuy, 2001; Mani et al., 2002). The TcdC protein is thought to encode an anti-sigma factor which sequesters the TcdR protein and prevents its association with the core RNA polymerase (Dupuy et al., 2008; Matamouros et al., 2007), although the role of TcdC in controlling toxin production is controversial (Bakker et al., 2012; Carter et al., 2011b; Cartman et al., 2012). The *tcdE* gene encodes a protein similar to a class of bacteriophage proteins known as holins (Tan et al., 2001). This suggests that TcdE may be a component of a novel holin-based mechanism responsible for toxin export in *C. difficile* (Tan et al., 2001). Although

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this hypothesis is supported by a recent study involving the analysis of a *tcdE* mutant in *C. difficile* strain JIR8094, which showed that toxin secretion from this strain was reduced in comparison to the wild-type strain (Govind and Dupuy, 2012), a similar independent study suggested that a *tcdE* mutation in strain 630Δerm did not reduce toxin secretion (Olling et al., 2012).

More recently, the LCT-encoding *tcsL* and *tcsH* genes of *C. sordellii* were shown to reside within a region similar to the *C. difficile* PaLoc (Sirigi Reddy et al., 2013). In addition to *tcsL* and *tcsH*, a gene encoding a bacteriophage holin-like protein named TcsE was identified, as was a gene that encodes a TcdR-family alternative sigma factor. Like TcdR in *C. difficile*, this protein, TcsR, was shown to be critical for LCT production in *C. sordellii* (Sirigi Reddy et al., 2013).

In addition to TcdR and TcsR from *C. difficile* and *C. sordellii*, respectively, TcdR-family proteins have also been identified in TpeL-negative strains of *C. perfringens*, where the UviA protein has been shown to control the production of a UV inducible bacteriocin (Dupuy et al., 2005). Similarly, BotR (Marvaud et al., 1998b) and TetR (Marvaud et al., 1998a) from *Clostridium botulinum* and *Clostridium tetani*, respectively, control the production of neurotoxins. Although these TcdR-family proteins are involved in expression of toxins, these toxins are not LCTs and the genes encoding these proteins do not appear to be associated with PaLoc regions.

The identification of a PaLoc-like region in *C. sordellii* raises the possibility that each of the LCT genes may reside within similar PaLoc-like regions and that these regions may encode the proteins needed to control the expression and export of these toxins. However, little is known about the genomic regions within which the *tpeL* and *tcnA* genes reside. In this study, we show that LCT production in *C. sordellii* and *C. perfringens* is repressed by glucose and is temporally regulated in a similar manner to TcdA and TcdB in *C. difficile*. We have also identified a previously uncharacterized PaLoc-like region in a TpeL-positive strain of *C. perfringens* and shown that the *tpeR* gene, which is located within this region, encodes a TcdR-family protein which is critical for TpeL production. Finally, we have shown that TpeR is functionally distinct from TcsR and TcdR from *C. sordellii* and *C. difficile*, respectively, despite belonging to the same protein family.

Materials and methods

Bacterial strains and culture conditions

The bacterial strains and plasmids used in this study are listed in Table 1. *Escherichia coli* strains were cultured aerobically in 2YT broth with agitation, or on 2YT agar (16 g tryptone, 10 g yeast extract, 5 g NaCl and 10 g agar in 1 l of distilled H_2O) at 37 °C. When selection was required, cultures were supplemented with chloramphenicol (25 μg/ml), tetracycline (10 μg/ml), or kanamycin (50 μg/ml). *C. perfringens*, *C. sordellii*, and *C. difficile* strains were cultured using supplemented heart infusion (HIS) broth or agar (Oxoid), containing 1.5% (w/v) glucose and 1% (w/v) L-cysteine hydrochloride at 37 °C under anaerobic conditions (10% H_2, 10% CO_2, 80% N_2) in a Don Whitley A35 Anaerobic Workstation. For selection, cultures were supplemented with D-cycloserine (250 μg/ml), erythromycin (10 μg/ml), or thiamphenicol (10 μg/ml), as required. For analysis of toxin production, clostridial strains were grown in tryptone yeast (TY) broth (30 g tryptone, 20 g yeast extract, 1 g sodium thioglycollate in 1 l distilled H_2O) or tryptone yeast glucose (TYG) broth (recipe as for TY broth with the addition of 1% (v/v) glucose). Growth of *C. perfringens* from frozen glycerol stocks was performed in fluid thioglycollate (FTG) medium prior to standard culturing procedures, as described above.

Isolation and manipulation of DNA

Plasmid DNA from *E. coli* strains grown overnight in 5 ml of broth, with appropriate antibiotic selection, was isolated using QIAprep spin miniprep columns (Qiagen) according to the manufacturer's instructions. *C. perfringens, C. sordellii* and *C. difficile* genomic DNA was prepared as previously described (O'Connor et al., 2006). DNA was quantified using a NanoDrop spectrophotometer (NanoDrop Technologies) and stored at −20 °C. Standard methods for the digestion, modification, ligation, and analysis of plasmid and genomic DNA and PCR products were used. Nucleotide sequence analysis was carried out using a Prism BigDye terminator cycle sequencing kit (Applied Biosystems) according to the manufacturer's instructions. Sequence detection was performed by Micromon at Monash University, and sequences were analyzed using ContigExpress (Invitrogen).

Genome sequencing

The genome of *C. sordellii* JGS6382 was sequenced using the Illumina HiSeq2000 platform and was performed at the Australian Genome Research Facility (AGRF). A total of 33,320,186-bp paired-end reads were generated, resulting in the assembly of 69 contigs. Genome assembly was then carried out using the Velvet software suite available at: http://www.ebi.ac.uk/~zerbino/velvet/.

Polymerase chain reaction (PCR)

For cloning purposes, DNA was amplified by PCR in reactions containing the following components: 10 μl of Failsafe Buffer E (Epicentre Biotechnology), 0.5 μl of each primer (50 μM), 0.5 μl of Phusion High-Fidelity DNA polymerase (NEB), and 100 ng of template DNA, in a final volume of 20 μl which was adjusted with dH_2O. Unless otherwise stated, the following PCR cycle conditions were used: initial denaturation at 94 °C for 4 min; followed by 30 cycles of denaturation at 94 °C for 30 s, annealing at 50 °C for 30 s, and extension at 72 °C for 1 min/kb of amplified DNA; a final extension step of 72 °C for 10 min was then performed. Splice overlapping extension PCR (SOE-PCR) was performed as previously described in the targetron user guide (Sigma-Aldrich), except that PCR was carried out using Phusion High-Fidelity DNA polymerase (NEB) and Failsafe Buffer E (Epicentre Biotechnology). All oligonucleotides used in this study are shown in Supplementary Table 1.

Construction of the tpeR and tcsR targetron plasmids

To insertionally inactivate the *tpeR* gene of *C. perfringens* and the *tcsR* gene of *C. sordellii*, Intron Finder (Carter et al., 2011a) was used to predict potential intron insertion sites and to design the oligonucleotide primers used for specific retargeting of the group II intron for the *tpeR* and *tcsR* genes. The primers used included DLP4, DLP153, DLP154 and DLP155 for *tpeR* and DLP4, DLP80, DLP81 and DLP82 for *tcsR*. Intron retargeting then was performed using splice by overlapping extension PCR (SOE-PCR). The 350-bp products generated were purified using the PCR purification kit according to the manufacturer's instructions (Qiagen) and then digested using restriction enzymes *Hind*III and *Bsr*GI. The digested fragments were then purified by extraction from a 0.8% agarose gel using a QIAquick Gel extraction kit according to the manufacturer's instructions (Qiagen). The purified fragments were then sub-cloned into the clostridial targetron plasmid pJIR3566 (Cheung et al., 2010), which had previously been digested with *Hind*III and *Bsr*GI. The resulting plasmids, pDLL31 (for *tpeR*) and pDLL5 (for *tcsR*), which carry introns retargeted to insert after nucleotide position 63 on the sense strand of DNA for *tpeR* and after nucleotide position 320 on the antisense strand for *tcsR*, were subsequently introduced into *E. coli*

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