BACTERIAL PATHOGENICITY

Molecular analysis of the promoter region of the *Clostridium difficile* toxin B gene that is functional in *Escherichia coli*

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Clostridium difficile is a human pathogen that produces two types of toxins, A and B, that cause a potentially lethal gastrointestinal syndrome termed pseudomembranous colitis. Virtually nothing is known about the mechanism of regulation of toxin production in this organism, and *cis*-regulatory regions of neither toxin have yet been identified, thus prompting this investigation. A motif homologous with the Shine-Dalgarno sequence of *Escherichia coli* occurs upstream from the putative initiation codon of toxin B, making this region also a candidate to contain a promoter. Therefore, a subgenomic DNA library of *C. difficile* in a plasmid vector was first constructed encompassing the 5'-end of the toxin B gene. A 450-bp DNA fragment was excised from the subgenomic DNA library clone and subcloned into a promoter-probe plasmid vector that contains two divergently oriented, promoterless genes to assay for promoter function. This subcloned DNA fragment directed the expression of alkaline phosphatase, a reporter gene product of the promoterless vector, thus indicating the presence of a functional promoter. To locate the promoter more precisely, a series of nested deletions of the toxin B promoter subclone was constructed with exonuclease III. The promoter that facilitates expression of the toxin B gene in *E. coli* was localised, based on alkaline phosphatase activity. The transcriptional initiation site of toxin B mRNA in *E. coli* was mapped by primer extension analysis, suggesting two closely associated tandem start sites directed by two similarly spaced promoters within this localised region.

Introduction

Clostridium difficile is a gram-positive, spore-forming, anaerobic bacillus. It is the aetiological agent of pseudomembranous colitis (PMC) in man, a severe, potentially lethal gastrointestinal disease that is characterised by a range of clinical presentation from a self-limited diarrhoea to an acute and fulminant colitis [1–3]. The development of the disease is a serious side-effect of treatment of patients with various antimicrobial agents. It is thought that *C. difficile* causes disease by producing two exotoxins, designated toxins A and B [4–7]. Toxin A is a potent enterotoxin that produces extensive tissue damage in the gut mucosa of tested animals. In addition to its enterotoxic activity, toxin A is also cytotoxic. Toxin B is a potent

cytotoxin, although it does not cause a significant response when given intragastrically to hamsters. However, it becomes lethal in combination with low doses of toxin A. This suggests that toxin A initiates tissue damage that provides toxin B with access to sensitive tissues. Both toxins are very high mol. wt proteins, and both appear to be produced in most toxigenic strains of *C. difficile*.

C. difficile strains can vary significantly in how much of both toxins they produce, but a 1:1 ratio is maintained by most strains, suggesting co-ordinated regulation [8, 9]. Although the genes for both toxins have been cloned and sequenced [10–12], thus far, the underlying molecular mechanism(s) that yield a balanced expression of both *C. difficile* toxin genes have not been elucidated. This is principally due to the inability to transform *C. difficile*. However, previous experiments by Dailey and Schloemer [13] and Barroso *et al.* [10] suggest that *Escherichia coli* may be a useful alternative organism to test for promoter activity of *C. difficile* genes.

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A candidate promoter for the toxin B gene is located between the presumed initiation codon of toxin B and the upstream *Xba*I site. DNA sequence analysis of this upstream region reveals a potential Shine-Dalgarno ribosome-binding site, AGGAG, 8-bp 5' to the putative initiation codon of toxin B gene. It is highly homologous to both the consensus sequence of *E. coli*, as well as those of clostridial strains, as summarised by Young *et al.* [14]. This prompted the current study on the identification and characterisation of a promoter for toxin B. The study showed that this DNA sequence has promoter activity in *E. coli*. The transcriptional start site of toxin B mRNA was also characterised, suggesting a location for the promoter site.

Materials and methods

Bacterial strains, media and plasmids

A highly toxigenic strain of *C. difficile* (strain VPI 10463) was obtained from Virginia Polytechnic Institute and State University in Blacksburg, VA, USA. The strain was grown anaerobically in Brain-Heart Infusion Broth (Difco Laboratories, Detroit, MI, USA). The *E. coli* strains used were DH5 α F'IQ (ϕ 80 dlacZ Δ M15, Δ (lacZYA-argF)U169, recA1, endA1, hsdR17 (r_K⁻, m_K⁺), supE44, λ ⁻, thi-1, gyrA, relA1, [F', proAB⁺, lacIqZ Δ M15, zff::Tn5(Km^r)] and CC118 (araD139, Δ (ara, leu)7697, Δ lacX74, phoA Δ 20, galE, galK, thi, rpsE, rpoB, argE(am), recA1). SOB medium (500 ml of water with tryptone 10 g, yeast extract 2.5 g NaCl 0.292 g and KCl 0.093 g) and WB buffer (glycerol 10% and distilled water 90%) were used for the electroporation procedure. The pQF110 plasmid vector, which contains a pair of divergently oriented, promoterless reporter genes (the alkaline phosphatase gene, *phoA*, and the luciferase gene, *lux*, one on each side of the multiple cloning region), was obtained from Dr Kropinsky [15] and was used as a promoter-probe vector. The plasmid pIBI25 (International Biotechnologies, New Haven, CT, USA) was used both in the construction of the subgenomic DNA library and as a cloning vehicle.

Molecular cloning, enzymes and DNA manipulations

The *E. coli* strain, DH5 α F'IQ, was used in the construction of a subgenomic DNA library, and CC118 was used in the alkaline phosphatase assay. Both were transformed with plasmid DNA and grown aerobically on LB agar plates or in LB broth [16] supplemented with ampicillin 40 mg/L when appropriate. Isolation of plasmid DNA from *E. coli* was performed by a modified boiling lysis method [17]. Bacterial genomic DNA was obtained from *C. difficile* grown to log phase. Restriction endonucleases, T4 polynucleotide kinase, T4 DNA ligase and Klenow fragments were purchased from Boehringer Mannheim

(Indianapolis, IN, USA), New England BioLabs (Beverly, MA, USA) and Promega Corporation (Madison, WI, USA) and they were used in accordance with the manufacturers' directions. 5-Bromo-4-chloro-3-indolyl phosphate (X-P) was from United States Biochemical (Cleveland, OH, USA). The exonuclease III assay was done with the Erase-a-Base System (Promega Corporation). SuperScriptTM RNAase H⁻ reverse transcriptase (BRL Life Technologies, Gaithersburg, MD, USA) was used in the primer extension assay. Unless otherwise stated, molecular techniques were used as described earlier [18].

Synthetic oligonucleotides

Synthetic oligonucleotides used for Southern blotting, DNA sequencing and primer extension analysis were either synthesised on a BioSearch 8700 DNA synthesiser (MilliGen/BioSearch, San Rafael, CA, USA) by the phosphoramidate method and purified by preparative polyacrylamide gel electrophoresis, or purchased from Integrated DNA Technologies (Coralville, IA, USA). The oligonucleotide primers used were as follows: (i) TOXB, 5'-TGAGTTTAGTTAATAGAAAA-3', (ii) Pri Ext, 5'-GGGACGAAATCTTACATT-3', and (iii) SEQ, 5'-GGATAAAAGTGCGCGTG-3'. Oligonucleotides were end-labelled with T4 polynucleotide kinase and [γ -³²P]-dATP (3000 Ci/mmol) (New England Nuclear, Boston, MA, USA).

Electroporation

The electroporation of *E. coli* cells was done with the Cell-PoratorTM Electroporation System and Cell-PoratorTM Voltage Booster from BRL Life Technologies, according to their recommendations. The electro-competent cells were prepared by growing 500 ml of cells in SOB medium with vigorous aeration at 37°C until an OD₅₅₀ of 0.8 was reached. The cells were then washed and resuspended in 500 ml of ice-cold sterile WB buffer twice. The cells were made competent by resuspension in 2 ml of WB buffer. Plasmid DNA was then mixed with 20 μ l of electro-competent cells and suspended in the 0.15-cm electrode gap Micro-Electroporation Chamber. The electroporation procedure was carried out with the pulse setting of 330- μ F capacitance, 4 k Ω resistance and 2.4 kV voltage, producing a time constant of *c.* 6 ms.

DNA sequencing

The DNA sequence was determined by the dideoxy-chain termination method of Sanger *et al.* [19] with labelled oligonucleotide primer, SEQ. The thermal cycle sequencing reactions were performed in a Coy 50 TempCycler (Coy Laboratory Products, Grass Lake, MI, USA) with thermostable Ventr[®](exo⁻) DNA polymerase (New England BioLabs). Sequencing reactions were done on denaturing polyacrylamide 5% gels [18].

Construction of a *C. difficile* subgenomic DNA library

The toxin B subgenomic DNA library of *C. difficile* was constructed by digesting *C. difficile* genomic DNA with *EcoRV* to completion. The digest containing the fragment of interest was separated by sucrose gradient centrifugation and the proper fraction identified by Southern blot analysis with an oligonucleotide probe, TOXB, which recognises the 5'-end of toxin B. This fraction was cloned in a pIBI25 plasmid vector prepared by *EcoRV* digestion (Fig. 1) and transformed into bacterial strain DH5 α F'IQ by electroporation. The colonies obtained were screened by the Grunstein-Hogness hybridisation technique [20] with the same oligonucleotide probe.

Cloning of a DNA fragment containing a putative promoter element

Clone B (Fig. 1) was digested with *Rsa*I, separated by agarose gel electrophoresis, and a 2.2-kb DNA fragment was electro-eluted, ligated to a *Sma*I-digested pIBI25 plasmid vector, and the product was transformed into *E. coli* strain DH5 α F'IQ. The resultant clone, pRStoxB (Fig. 1), was then digested with *Xba*I to release a fragment of 460 bp. This DNA fragment was subcloned into the *Xba*I site upstream of a promoterless alkaline phosphatase gene of *E. coli* vector pQF110 [15]. The resultant recombinant clone, pPB (Fig. 1), was transformed into an alkaline phosphatase-deficient bacterial host, CC118, and examined for alkaline phosphatase activity. The portion of the toxin B structural gene in the 460-bp insert DNA was in the same polarity as the alkaline phosphatase gene in pQF110 vector (Fig. 1). The junctional regions of the clones were confirmed by DNA sequencing.

Alkaline phosphatase assays

The alkaline phosphatase activities were assayed by both screening qualitatively for blue bacterial colonies grown overnight at 37°C on LB plates with X-P 40 μ g/ml, and quantitatively by measuring spectrophotometrically the rate of *p*-nitrophenyl phosphate hydrolysis at OD₄₂₀ by permeabilised cells according to Manoil and Beckwith [21].

Construction of nested deletions of the promoter

The pPB promoter clone DNA was prepared by digesting with both *Kpn*I and *Bam*HI enzymes and then exonuclease III at 20°C from 0.5 min to 8 min at 30-s intervals, followed by S1 digestion, Klenow treatment, ligation and transformation into CC118 bacterial host. The S1 nuclease digestion was done at 4°C and with 500 mM NaCl to more rigorously control the digestion. The progressive deletion of pPB clone was initiated from the *Bam*HI site towards the putative promoter region of toxin B. Colonies were selected for each time point, and the plasmid DNA was prepared for

further restriction enzyme characterisation by both *Bss*HII and *Hind*III, and analysed on a high resolution agarose 1.5% gel. The authenticity of each deletion clone was verified by DNA sequencing.

Isolation of RNA

Whole-cell RNA was extracted from cultures of *E. coli* as described by Reddy and Gilman [22] with slight modifications. Briefly, a 100-ml culture of *E. coli* was grown to an optical density of 0.8 at 550 nm. The cells were then resuspended in STET lysing buffer (sucrose 8%, Triton X-100 5%, 50 mM EDTA, 50 mM Tris HCl, pH 7.0), and extensively vortex mixed in phenol/chloroform reagent. The nucleic acid was precipitated with ethanol, and dissolved in diethyl pyrocarbonate (DEPC)-treated water. The phenol/chloroform extraction was repeated twice and the final nucleic acid pellet was dissolved in 9 ml of DEPC-treated water containing 4.5 g of CsCl, layered over a 3-ml cushion of 5.7 M CsCl, and centrifuged at 30 000 rpm for 24 h at 20 °C in a Beckman SW41 rotor. The supernate was discarded after centrifugation and the RNA pellet was dissolved in DEPC-treated water, and precipitated with ethanol again. Purified RNA was dissolved in 200 μ l of DEPC-treated water, and its concentration was determined by absorbance at 260 nm.

Primer extension of mRNA

Primer extension was performed on whole-cell RNA extracted as above by a modified procedure of Triezenberg [23]. The annealing of the radiolabelled primer, PriExt, to the RNA was done at 80°C for 3 min, followed by cooling of the sample to 37°C for a further 30 min. The primer extension reaction was performed at 42°C for 1 h without actinomycin D, and the product was analysed on a polyacrylamide 9% gel.

Results

Identification of a promoter region in clone pPB

Clone B (Fig. 1) from the subgenomic DNA library contained an 8.8-kb *EcoRV*-digested DNA fragment, which included 3.8 kb of the 5'-end of the toxin B gene that was previously sequenced [10], and another uncharacterised 5 kb of DNA upstream from the toxin B gene. The DNA of clone B was assayed for promoter activity with the promoterless plasmid vector, pQF110 [15]. The resultant recombinant clone, pPB (Fig. 1), contained 400 bp of DNA sequence upstream from the initiation codon of toxin B. When this clone was tested on LB plates with X-P, it displayed a blue colour phenotype. The parental plasmid, pQF110, produced only white colonies, indicating that the blue recombinant clones exhibited a promoter activity resulting from the cloned insert. Quantitative analysis (Table 1) of the

Fig. 1. pPB clone construction for functional analysis. Clone pPB was constructed as described in *Materials and methods*. The 2.2-kb *RsaI*-generated DNA fragment in clone B contains 2.15 kb of DNA upstream from the proposed initiation codon [10] of toxin B gene and *c.* 50 bp of 5'-end toxin B structural gene. This fragment was excised and cloned into the *SmaI* site of pBI25 vector, creating clone pRStoxB. The 460-bp *XbaI*-digested DNA fragment from pRStoxB contains 398 bp of DNA upstream from the proposed initiation codon of toxin B, 54 bp of toxin B structural gene and 8 bp of flanking pBI25 vector DNA sequence. Both the reporter genes of pQF110 plasmid vector are shown as dark boxes: *phoA* for alkaline phosphatase and *lux* for luciferase. MCS is the multiple cloning site of pQF110. S/R and R/S are the junctional regions created by ligating together *SmaI* with *RsaI* ends. ■ the region of the *C. difficile* DNA not previously sequenced; ■, the Shine-Dalgarno sequences in the pQF110 vector and in the putative sequence for toxin B gene; ➤ the putative promoter for toxin B gene; the translational stop codons in all three reading frames present in pQF110 are shown, (U). Restriction endonucleases are: B, *Bam*HI; E, *Eco*RV; H, *Hind*III; K, *Kpn*I; N, *Nco*I; R, *Rsa*I; S, *Sma*I; T, *Tth*III; X, *Xba*I. This figure is not drawn to scale for simplicity of display.

promoter activity in clone pPB with *p*-nitrophenyl phosphate showed significant alkaline phosphatase activity compared to the parental clone, pQF110, thus confirming the earlier results from the X-P phenotypic analysis that the cloned DNA fragment indeed contained promoter activity.

Table 1. Quantitative analysis of alkaline phosphatase expression in the promoter-related clones by the *p*-nitrophenyl phosphate assay

Clones and deletion clones	Alkaline phosphatase activity (units)*	Fold increase
CC118	0.1	0
pQF110	0.4	0
pPB	66.1	165
pPB16	279.6	699
pPB17	607.1	1518
pPB18	627.1	1568
pPB27	16.1	40
pPB71	6.6	17
pPB83	3.9	10

*Alkaline phosphatase activity of each clone, as measured spectrophotometrically at OC₄₂₀ by the *p*-nitrophenyl phosphate assay and expressed in units as defined by Beckwith and Manoil [21].

Localisation of the promoter region by nested deletion analysis

To localise the position of the putative promoter in clone pPB, a set of nested deletions of the clone was performed with an exonuclease III assay. Six of the clones shown (Fig. 2), represented deletions from 79 bp to 232 bp, and they were analysed for functional promoter activity. The results of the alkaline phosphatase phenotype characterisation of these deletion clones are also shown in Fig. 2, where deletion clones pPB27, pPB71 and pPB83 conferred a white colony phenotype, and three others, pPB16, pPB17 and pPB18, conferred a blue phenotype. The alkaline phosphatase activity of the promoterless vector disappeared with a transition from deletion clone pPB18 to pPB27, implying that essential promoter function exists within this deleted region. A quantitative assay with *p*-nitrophenyl phosphate showed that clones pPB16, pPB17 and pPB18 exhibited much higher alkaline phosphatase activity than the other three clones, pPB27, pPB71 and pPB83. These results were consistent with the phenotypic analysis of the nested deletion clones with X-P (Fig. 2).

Primer extension analysis to localise the start site of toxin B transcript

The transcriptional initiation site of the toxin B mRNA was identified by primer extension analysis with the 5'-end-labelled primer, PriExt, and total RNA extracted from clone pPB. Two product bands were observed (Fig. 3), indicating that the 5'-end of the toxin B mRNA was produced by two different promoters. Control experiments with either the RNA extracted from cells carrying the parental pQF110 vector, or adding RNAase prior to primer extension analysis did not show any product (Fig. 3). Therefore, the primer extension initiated from the mRNA encoded by the cloned DNA fragment. The longer transcript was mapped to an adenine residue which was 15 bp upstream of the presumed toxin B start codon [10] (Fig. 4), and the shorter transcript started at a guanine residue 9 bp upstream of the toxin B start codon [10],

Fig. 2. Nested deletion analysis of clone pPB. The six deletion clones are shown with the amount of deletion measured from the *Kpn*I site to the last nucleotide removed by *exo*III. The relative positions of the putative Shine-Dalgarno (SD) sequence and the putative promoter region (the -35 and -10) are indicated. The blue and white phenotypes of the deletion clones are shown as ■, or □, respectively. ■, represents the region of the cloned insert DNA containing 54 bp of the 5'-end of the structural toxin B gene, beginning from the originally proposed initiation codon [10], and 8 bp of the flanking pIBI25 vector sequence, as described in Fig. 1. Restriction enzymes are: B, *Bam*HI; K, *Kpn*I; X, *Xba*I; S/R, junctional region between *Sma*I and *Rsa*I sites.

Fig. 3. Primer extension analysis of clone pPB. This was performed as described in *Materials and methods* and the two products, P1 and P2, were analysed on a denaturing polyacrylamide 9% gel. Lane 1, RNA from clone pPB; 2, RNA from clone pQF110; 3, RNA from clone pPB, but treated with RNAase before primer extension. The numbers to the right indicate the positions of the relevant DNA size marker generated by digesting pIBI25 plasmid with *Sau*3A.

which was also the last base of the 3'-end of the putative Shine-Dalgarno sequence (Fig. 4).

Discussion

This study aimed to determine the location of the promoter for the toxin B gene of *C. difficile*, a

clinically relevant organism whose toxins are believed to be the principal virulence factors in the enteric disease, pseudomembranous colitis. The study is necessarily done with *E. coli*, as *C. difficile* has not yet been successfully transformed with exogenous DNA. Based on the analysis of clone pPB (Fig. 1), promoter activity was found within the 400-bp DNA fragment upstream from the putative start codon of the toxin B gene. However, it is noted that the authentic initiation codon of the toxin B gene is not yet known, whether it is expressed in *E. coli* or *C. difficile*. Subsequent deletions of clone pPB, followed by sequence analyses, also showed that if > 38 bp upstream of the presumed toxin B gene initiation codon are deleted, promoter activity would be lost, yet it would not be affected if only 11 bp of DNA are deleted (Fig. 2). This indicates that an essential part of the promoter occurs within the 11–38-bp region upstream of the presumed start codon of the toxin B gene.

Also, as noted in Table 1, clone pPB exhibits 4.2–9.4-fold less promoter activity than three of the deletion clones (pPB16, pPB17 and pPB18), as measured by the *p*-nitrophenyl phosphate assay. A possible explanation for this phenomenon is the presence of an inhibitory factor acting either at the transcriptional or translational levels to reduce the amount of the alkaline phosphatase activity being synthesised. It is not known why the alkaline phosphatase activity of clone pPB16 is lower than that of clones pPB17 and pPB18, although this is a very reproducible phenomenon. We speculate that this may be related to a less optimal positioning of the Shine-Dalgarno sequence in clone pPB16 compared to these other two clones, thus possibly reducing the efficiency of translation of the alkaline phosphatase product of clone pPB16. Never-

Fig. 6. Model of the putative toxin B dual promoter region expressed in *E. coli*. The two putative ribosome-binding sites (Shine-Dalgarno) are boxed, where the dashed box represents an alternative ribosome binding site suggested here for toxin B. The first nucleotide for each *exoIII* deletion clone is indicated by an inverted arrow above the sequence with the name of the clone, where the open arrow, ∇ , indicates no promoter activity and the filled arrow, $\blacktriangledown$, indicates promoter activity. The asterisk by P1 or P2 designates the first base of the longer and shorter transcript, respectively, from primer extension analysis. Both sets of '-35' and '-10' putative promoter elements are overlined; the bold face type for the longer transcript, and the plain face type for the shorter transcript. The amino acids for toxin B are listed below the respective codons. The alternative initiation codon, **M**, for toxin B suggested here is marked with bold face type.

However, it remains to be determined which initiation codon is used *in vivo* by *C. difficile*.

These two proposed promoters for toxin B not only fit the current models of *E. coli* and gram-positive [26, 27] consensus promoter sequences, but they also find additional support from gram-positive organisms in the -45 region of the promoter (Fig. 5). Further support for this model comes from clone pPB27 (Fig. 6), which does not contain both proposed -10 regions of this double promoter, and therefore would not be expected to express the alkaline phosphatase gene, as is actually observed (Fig. 2). In addition, other deletion clones, which either contain all the essential promoter elements known for *E. coli*, or none, display an alkaline phosphatase activity (Fig. 2) consistent with this double promoter model (Fig. 6).

If both promoters are used *in vivo* for *C. difficile*, a

sporulating gram-positive bacterium, their purpose remains to be determined. This may be related to unique features of the toxin B gene and its function in *C. difficile*, and the complex regulation anticipated for such toxin genes, whose expression correlates with sporulation [29]. For example, *Bacillus subtilis*, a well-characterised, sporulating gram-positive organism, utilises various σ factors with different promoter specificities during sporulation. One of its genes, *spoVG*, contains two closely overlapping promoters, whose transcription during the different developmental stages of sporulation is controlled by at least two different σ factors of the RNA polymerase [30]. Therefore, the promoter activity of the toxin B gene in *C. difficile* may be under similarly complex regulation, which is absent in *E. coli*. This may involve some unique σ factors and perhaps other regulatory factors, thus offering very interesting opportunities for further work.

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