INFECTION AND IMMUNITY, Mar. 1997, p. 1105–1108 Vol. 65, No. 3
0019-9567/97/$04.00+0

Positive Regulation of *Clostridium difficile* Toxins

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Received 13 August 1996/Returned for modification 31 October 1996/Accepted 9 December 1996

The toxigenic element of *Clostridium difficile* VPI 10463 contains a small open reading frame (ORF) immediately upstream of the toxin B gene (G. A. Hammond and J. L. Johnson, Microb. Pathog. 19:203–213, 1995). The deduced amino acid sequence of the ORF, which we have designated *txeR*, encodes a 22-kDa protein which contains a helix-turn-helix motif with sequence identity to DNA binding regulatory proteins. We used a DNA fragment containing the *C. difficile* toxin A repeating units (ARU) as a reporter gene to determine if *txeR* regulates expression from the toxin A and toxin B promoters in *Escherichia coli*. To test the affect of *txeR* on expression, we fused the ARU gene fragment in frame with the toxin promoters. The fusions expressed a 104-kDa protein that contained the epitopes for monoclonal antibody PCG-4, which we used to measure levels of recombinant ARU by enzyme-linked immunosorbent assay. When *txeR* was expressed in *trans* with the toxin B promoter-ARU fusion contained on separate low-copy-number plasmid, expression of ARU increased over 800-fold. Furthermore, when we tested the toxin A promoter fused to ARU, expression increased over 500-fold with *txeR* supplied in *trans*. Our results suggest that TxeR is a positive regulator that activates expression of the *C. difficile* toxins.

Treatment with antibiotics is commonly accompanied by gastrointestinal complications, particularly among elderly hospitalized patients. Symptoms range from mild diarrhea to severe pseudomembranous colitis. An estimated 25% of antibiotic-associated diarrhea is attributable to *Clostridium difficile*. Almost all cases of pseudomembranous colitis, however, are caused by this spore-forming anaerobe. Despite available treatment, *C. difficile* continues to be one of the most important and costly nosocomial pathogens. Indeed, the incidence of the disease is increasing due to continued widespread and sometimes inappropriate use of broad-spectrum antibiotics (3, 4, 20, 22).

The production of two potent protein toxins by *C. difficile* is responsible for the intestinal damage of the disease. The toxins, termed A and B, are the largest known bacterial toxins, with molecular weights of 308,000 and 269,000, respectively. Toxin A is enterotoxic, while toxin B has little or no enterotoxic activity. Both toxins are lethal and cytotoxic, although toxin B is a more potent cytotoxin (20, 22, 28). The toxin genes have been sequenced (2, 7), and the deduced amino acid sequences contain 49% identity and 63% similarity when conserved substitutions are considered, suggesting that the toxins arose by gene duplication (32, 33). Moreover, the proteins share a number of similar structural features, including a putative nucleotide binding site, a central hydrophobic region, four conserved cysteines, and a long series of repeating units at their carboxyl ends (1, 32, 33). In the case of toxin A, the repeating units are immunodominant and are responsible for binding to type 2 core carbohydrate antigens on the surface of the intestinal epithelium (18, 31). Additionally, the toxins share a mechanism of action involving the catalytic transfer of a glucose from UDP-glucose to the low-molecular-weight Rho proteins which function in organization of the actin microfilaments (6, 16, 17). The covalently modified Rho proteins are unable to maintain cytoskeletal organization, resulting in the characteristic cell rounding caused by the toxins.

Both toxins are found in the stools of patients with the disease, and each is probably involved in the pathology of the disease. Toxigenic strains, with extremely rare exceptions, produce both toxins (5, 19, 30). Furthermore, the toxins appear to be coordinately regulated, as strains which produce high levels of toxin B also produce high levels of toxin A. Toxin production, however, varies as much as 10^5-fold among toxigenic isolates (12, 22).

The toxin genes are located next to each other, with a small open reading frame (ORF) in between. Hammond and Johnson recently defined the region of the *C. difficile* VPI 10463 chromosome containing the toxin genes as the "toxigenic element" (12). In addition to the toxin genes, the 19.6-kb region contains three small ORFs. The small ORFs are not contained by nontoxigenic strains, which suggested to us that they might be involved in virulence. One of the ORFs, which we have designated *txeR*, is located immediately upstream of the toxin B gene.

The *txeR* gene encodes a small (M_r of ~22,000) basic protein of 184 amino acids with an unusually high content of lysine residues within its C-terminal region. A motif search of the PIR protein data libraries (DNAStar, Inc. Madison, Wis.) by using software program MacPattern (Fuchs) identified a helix-turn-helix motif sequence with identity to DNA binding bacterial response regulators. Moreover, the database search revealed that TxeR contains sequence identity with UviA, a putative positive regulator of bacteriocin production in *C. perfringens* (10, 11). TxeR also contains sequence identity with Orf-22 of *C. botulinum* C 468, a putative positive regulator of botulinum neurotoxin C1 (14). Alignment with the deduced amino acid sequence of TxeR revealed 28% identity with UviA and 21% identity with Orf-22 (Fig. 1).

Promoter-like structures of the toxin genes have been identified. They are located distal (approximately 200 nucleotides) to the start codons and have 76% sequence identity in the −10 to −35 region (32). Primer extension analysis of the toxin gene transcripts indicates that these are indeed functional promoters of the *C. difficile* toxin genes (13). The −35 regions of the

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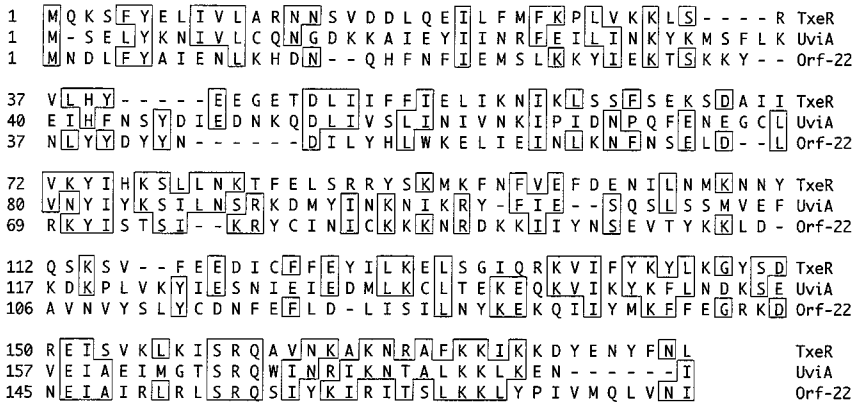

FIG. 1. Alignment of TxeR with UviA and Orf-22. Identical amino acids are boxed. The helix-turn-helix motifs within the putative DNA binding regions correspond to TxeR amino acids 149 to 168, UviA amino acids 156 to 175, and Orf-22 amino acids 144 to 163.

toxin promoters are similar to those of the UV-inducible promoters of *C. perfringens* bacteriocin production (11).

Toxin promoter-ARU fusions. To measure expression levels from the toxin promoters, we constructed vectors containing each of the toxin promoters fused to the toxin A repeating units (ARU). A schematic of the *C. difficile* VPI 10463 toxigenic element and the DNA fragments used to construct the toxin promoter-ARU fusions is shown in Fig. 2. The toxin promoters and ORF of *txeR* were amplified by PCR using primers with terminal restriction sites at the 5′ end to facilitate subcloning. Primers for the PCRs were as follows: *txeR* ORF, 5′-AGGGTGATCATATGCAAAAGTCTTT-3′ and 5′-CTCTGCAGTTACAAGTTAAAATAAT-3′; toxin B promoter, 5′-CGCGAATTCTCTAGACAAGCTGTTAATAAGGC and 5′-CGCGGATCCCTCATAAAATTTTCTCCTTTAC-3′); and toxin A promoter, 5′-TGATCTAGATGCTAAGGATGAAAAG-3′ and 5′-GGGGATCCGACATAAAAACCTCTTAGTAT. Restriction sites in the primers are underlined.

A *Bam*HI site was placed immediately downstream of the start codon of the toxin genes to allow in-frame insertion of the reporter gene ARU. The restriction fragment encoding ARU contained the final 2,601 bp of the toxin A structural gene and downstream transcriptional termination signal. The gene fusions expressed a 104-kDa protein, as verified by immunoblotting (data not shown) with monoclonal antibody PCG-4, which binds to epitopes in the ARU (9).

To express *txeR* in *trans*, we amplified its ORF and subcloned the PCR product to the expression vector pT7-7. Restriction sites were included at the 5′ end of the primers to facilitate in-frame insertion in the T7 expression vector. The resulting vector, pT7-*txeR*, expressed *txeR* under control of the T7 promoter.

Previously described plasmids pCDtoxB and pCDtoxA.03 were the source for the toxin promoters and the ARU reporter gene fragment (15, 26). Plasmid pCD21, used for amplification of the *txeR* ORF, was a generous gift from J. L. Johnson (Department of Biochemistry, Virginia Polytechnic Institute and State University, Blacksburg). The PCRs were performed with high-fidelity Ultma polymerase and nucleotides purchased from Perkin-Elmer Cetus (Norwalk, Conn.). All primers used for amplifications were generated by DNAgency (Malvern, Pa.). Methods for DNA manipulations were as described by Sambrook et al. (27). All DNA fragments, including digested PCR products, were isolated from 0.7% agarose gels with an Elu-Quick DNA purification kit (Schleicher & Schuell, Keene, N.H.) following electrophoresis.

Positive regulation of the toxin B promoter by *txeR* in *trans*. To allow the expression of *txeR* in *trans*, we subcloned the toxin B promoter-ARU fusion to the low-copy-number plasmid pACYC184, which can coexist with the pUC-based pT7-*txeR* vector. To determine the effect of the *txeR* gene product on expression from the toxin B promoter, we cotransformed *Escherichia coli* BL21(DE3) with pT7-*txeR* and pACYC184 plasmid containing the toxin B promoter-ARU fusion (pAC-BP-ARU). Additionally, to determine expression levels in the absence of the *txeR* gene product, BL21(DE3) was cotransformed with pT7 and the plasmid containing the toxin B promoter-ARU fusion.

The level of ARU in recombinant lysates was determined with the ToxA Test (TechLab, Inc., Blacksburg, Va.), which uses PCG-4 horseradish peroxidase as the detecting antibody (21). Units of enzyme-linked immunosorbent assay reactivity were arbitrarily defined as the A_{450} multiplied by the reciprocal of the highest dilution with an absorbance of 0.2 to 2.3. The

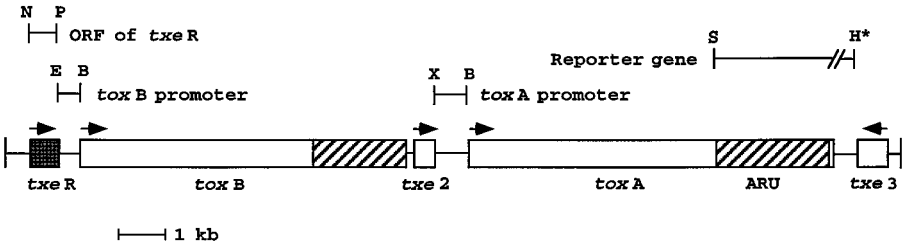

FIG. 2. Toxigenic element of *C. difficile* VPI 10463 and strategy used for construction of expression vectors. The hatched boxes represent the repeating units of the toxins. Arrows indicate the direction of transcription. DNA fragments derived from the toxigenic element are shown at the top. Restriction sites: N, *Nde*I; P, *Pst*I; E, *Eco*RI; B, *Bam*HI; H, *Hin*dIII; Sa, *Sau*3a; X, *Xba*I; H*, *Hin*dIII restriction site from the pUC19 vector.

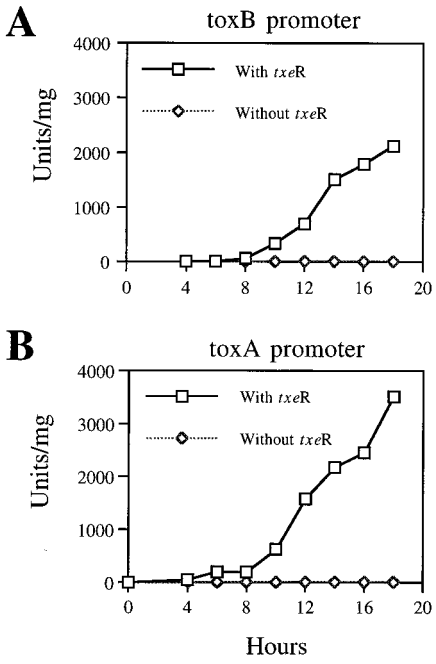

FIG. 3. Time course of the effect of *txeR* in *trans* on expression from the toxin promoters.

level of ARU is expressed as units per milligram of total protein in the recombinant lysate. No reactivity was observed in undiluted samples of control strain BL21(DE3) harboring pACYC184 and pT7. Recombinant strains were grown at 37°C in Terrific broth (29) supplemented with ampicillin (100 μg/ml) and chloramphenicol (100 μg/ml).

The level of ARU expression with and without *txeR* expressed in *trans* was followed over an 18-h time course. Expression of ARU from the toxin B promoter gradually increased over time with *txeR* expressed in *trans* (Fig. 3A). Levels began to rise significantly at about 8 h and continued to rise during stationary phase (growth curve not shown). By 18 h, the level of ARU expressed in the presence of *txeR* was over 800-fold higher than that without *txeR* (Table 1). Expression of *txeR* was not induced; therefore, leaky expression from the T7 promoter was sufficient for *trans* activation of the promoter.

Positive regulation of the toxin A promoter in *trans*. To determine if *txeR* also positively regulated expression from the toxin A promoter, we performed similar experiments with the toxin A promoter-ARU fusion expressed from pACYC184 in BL21(DE3). Expression of ARU from the toxin A promoter increased gradually with *txeR* supplied in *trans* (Fig. 3B). As with the toxin B promoter, significant activation of expression did not occur until cultures approached stationary phase (growth curve not shown). By 18 h, the level of ARU expression was over 500-fold higher with the pT7-*txeR* vector present (Table 1).

TABLE 1. Expression levels of ARU (18 h)

txeR supplied	Promoter	ARU level	
		U/mg	Fold increase
No	Toxin B	2.4	0
In trans	Toxin B	2,120	883
No	Toxin A	6.2	0
In trans	Toxin A	3,516	567

The study of gene regulation in *C. difficile* is hampered by limited methods of genetic exchange. Genes have been introduced on the chromosome by conjugal mating. Gene transfer, however, required *Bacillus subtilis* as an intermediate host (23). In this study, we were able to identify a positive regulator of the toxin genes with experiments using *E. coli*. A detailed understanding of how the toxins are regulated, however, will require genetic studies of *C. difficile*.

It is not known what triggers the production of toxins by *C. difficile* when the normal gut flora is disrupted by antibiotics. One likely possibility is that the organism is simply allowed to grow to large enough numbers in the absence of competing organisms and in the process produces sufficient amounts of the toxins to cause disease. On the other hand, stressful events such as increased oxygen tension, elevated temperature, limited nutrients, and the presence of small amounts of antibiotics have been observed to increase toxin production in vitro (24, 25, 34). *C. difficile* produces large amounts of both toxins when grown in rich media in dialysis tubing (22). When the organism is grown in the same rich media in free culture, however, very little of the toxins are produced. The difference in toxin production is probably due to nutrient limitation controlled by the diffusion rate across the membrane. This results in slow growth and an extended stationary phase in the dialysis culture (8). A similar situation of nutrient limitation is likely encountered by *C. difficile* in the gut, leading to toxin production activated by TxeR.

We thank Elizabeth A. Pennell for excellent technical assistance.

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Editor: A. O'Brien