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Construction and Expression of the Complete *Clostridium difficile* Toxin A Gene in *Escherichia coli*

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Cloned fragments constituting the 8.1-kb toxin A gene of *Clostridium difficile* were used to reconstruct the intact gene. The recombinant toxin expressed in *Escherichia coli* was cytotoxic, enterotoxic, and lethal. In addition, toxic lysate caused hemagglutination of rabbit erythrocytes. The toxic activities were inhibited by antibody specific for toxin A. Our findings demonstrate that the biological activities exhibited by native toxin A are functions of a single protein encoded by the 8.1-kb toxin A gene, independent of any other *C. difficile* gene products.

Toxigenic strains of *Clostridium difficile* cause the antibiotic-associated disease pseudomembranous colitis (2, 3, 6). Two large toxins, A and B, are produced by the organism (1, 14). In animal studies, toxin A produces extensive tissue damage in the gut mucosa (7, 11). Toxin B, a potent cytotoxin, is inactive in the intestine when administered orally by itself but becomes lethal in combination with low doses of toxin A (8). This suggests that toxin A initiates tissue damage that provides toxin B with access to sensitive tissues. The mechanisms of action of these toxins are unknown, but effects on tissue culture cells suggest they may act in a similar fashion.

Some insight into the nature of the toxin A protein has been gained from DNA sequencing data published by Dove et al. (4). We now know that the toxin is a large molecule (M_r of 308,000) and that it lacks a leader sequence at the amino terminus. Nearly one-third of the protein is part of a complex binding moiety composed of numerous repetitive amino acid sequences. To this point, however, none of the gene fragments used for sequencing have produced a toxic product. Data base searches have failed to uncover any meaningful similarities with other proteins or nucleic acids that might reveal the toxin's mode of action. As a result, we still have little insight into the region of the molecule responsible for toxicity or into the actual mode of toxic activity.

Several groups of investigators have reported the cloning of toxin A gene fragments. Price et al. (13) reported the cloning of a 4.7-kb fragment coding for a receptor-binding region of toxin A. Muldrow et al. (12) described a peptide encoded by a 0.3-kb gene fragment that reacted with toxin A antibody. Von Eichel-Streiber et al. (17) cloned a series of overlapping gene segments and reported that peptides expressed from these fragments were reactive with toxin A antibody. None of these groups reported toxicity associated with their expressed peptides. Wren et al. (18) reported that a portion of a 14.3-kb insert cloned into bacteriophage vector EMBL3 coded for *C. difficile* toxin A. The expressed product, which had an M_r of 235,000, exhibited hemagglutinating and cytotoxic activities. No data on enterotoxicity were presented.

Using recombinant DNA techniques and previously cloned toxin A gene fragments, we have successfully reconstructed the entire *C. difficile* toxin A gene in *Escherichia coli*, in the absence of any other clostridial genes. To do this, we have taken advantage of toxin A sequencing clones and a well-defined restriction map of the gene and regions flanking it. The protein expressed by this gene is toxic and is neutralized by antiserum to *C. difficile* toxin A. All the toxic activities attributed to native toxin A are associated with the peptide expressed from the 8.1-kb toxin A gene.

MATERIALS AND METHODS

Enzymes and reagents. Restriction endonucleases, T4 ligase, exonucleases III and VII, and the Klenow fragment of DNA polymerase I were purchased from Bethesda Research Laboratories, Inc., Gaithersburg, Md. All enzymes were used as recommended by the manufacturer. SeaPlaque, a low-gelling-temperature agarose, was purchased from FMC Bioproducts, Rockland, Maine. All ligations in this study were performed with fragments separated by electrophoresis and excised from the gel. Isopropyl-β-D-galactoside (IPTG) was purchased from Sigma Chemical Co., St. Louis, Mo. Reagents for dideoxy sequencing of the gene, including Sequenase enzyme, were purchased from United States Biochemical, Cleveland, Ohio. Oligonucleotide primers were purchased from Thomas Reynolds, Medical College of Virginia, Richmond. DEAE-Sepharose CL-6B used for purification of the recombinant toxin was purchased from Sigma. Affinity-purified antibody to *C. difficile* toxin A was prepared as previously described (9).

Bacteria and plasmids. All cloning was done in *E. coli* JM109 (Bethesda Research Laboratories) and χ1776 (ATCC 31244; American Type Culture Collection, Rockville, Md.). χ1776 is an EK2/BL2-approved host, and all toxic peptide expression in JM109 was done under BL3 biological containment. The cloning of pCD17 and pCD11R-6 has been described (4). Clone pCD19D/P was constructed by the insertion of an 867-bp *Dra*I-*Pst*I fragment from pCD19 (4) into the *Sma*I-*Pst*I sites of pUC18. This fragment encompasses the first 798 bases of the toxin A gene as well as a short segment preceding the gene. The pUC plasmids used in this study were purchased from Bethesda Research Laboratories. The methods for purified plasmid preparation have been described by Maniatis et al. (10).

Transformed JM109 cells were plated on LB agar plates (10) supplemented with 100 μg of ampicillin per ml. Trans-

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formed χ1776 cells were plated on χ1776 agar (10) supplemented with ampicillin. Broth cultures were grown at 37°C in TB broth (15) containing 100 μg of ampicillin per ml. χ1776 broth cultures were supplemented with 0.1 M $MgCl_2$, 0.5% diaminopimelic acid, and 0.2% thymidine.

Crude culture filtrates of *C. difficile* 10463 were prepared as described by Sullivan et al. (14).

Protein concentration determination. Protein concentrations were determined by using the Coomassie Protein Assay Reagent, purchased from Pierce Chemical Co., Rockford, Ill., following the instructions provided by the manufacturer. Bovine serum albumin was used as the protein standard.

Electroporation. Transformation of bacterial cells was performed with a Transfector 100 electroporator purchased from BTX, Inc., San Diego, Calif. Competent *E. coli* cells were prepared by the method of Dower et al. (5) and frozen in a solution of 10% glycerol in water. An output voltage of 650 V and a 5-ms pulse were used, with an electrode gap of 0.5 mm.

Construction of the toxin A gene. The strategy used for the cloning of the intact toxin A gene is illustrated in Fig. 1a. Three restriction fragments excised from clones used for sequencing were ligated to reconstruct the gene. The 1.7-kb insert of pCD17 was inserted behind the 0.9-kb insert of pCD19D/P. The new clone, carrying the first 2.5 kb of the toxin A gene as well as 69 bases preceding the gene, was designated pCD19/17. The inserted fragment of pCD19/17, along with the 6.8-kb *Hin*dIII-*Hin*cII fragment of the insert in clone pCD11R6, was ligated with the plasmid vector to produce pCDtoxA.6. The 9.4-kb construct resulting from the fusion of these three fragments contains the toxin A gene as well as a 695-bp open reading frame (ORF) that follows the toxin gene and reads in the direction opposite that of the toxin A gene.

The pCDtoxA.6 insert was digested from the 3′ end by the procedure described by Yanisch-Perron et al. (19), but with the exonuclease III incubation time extended to 90 min and aliquots collected at 10-min intervals. All but the last 33 bases of the ORF at the 3′ end of the toxin A gene were excised. This new clone, containing an 8.6-kb insert, was designated pCDtoxA.03. The alignment of the recombinant clone fragments in this construct is illustrated in Fig. 1b. Correct alignment of the gene fragments and the extent of base excision in pCDtoxA.03 were monitored by restriction enzyme digestion and sequencing across the fragment junction sites.

Expression of recombinant peptide. Recombinant *E. coli* broth cultures (250 ml in 1-liter flasks, rotated at 200 rpm) were grown at 37°C to an optical density of 1.0 at 600 nm, followed by the addition of IPTG to a final concentration of 2.0 mM to induce the *lacZ* promoter of the pUC vector. After 2 h of continued incubation, the cells were centrifuged and then resuspended in 0.05 M Tris (pH 7.4) containing 0.2 M NaCl (TBS) to yield a 40× concentration of the original culture volume. Resuspended cells were subjected to six 10-s pulses of sonication with a sonicator (Ultrasonics Inc., Farmingdale, N.Y.) equipped with a microtip probe operated at 40% power. Cell debris was removed by centrifugation, and the resulting supernatant was passed through a 0.2-μm-pore-size filter. Cultures grown without IPTG induction were grown for 16 h to maximize recombinant peptide production and then treated as described above for cell lysis.

Purification of recombinant peptide. Ten milliliters of lysate containing the expressed recombinant peptide was dialyzed extensively against 0.05 M Tris-HCl buffer (pH 7.5). The dialyzed preparation was applied to a column containing 10 ml of DEAE-Sepharose CL-6B, and the gel was washed with 10 bed volumes of the Tris buffer. Bound recombinant peptide was eluted with 2 bed volumes of 0.05 M Tris-HCl buffer (pH 7.5) containing 0.15 M NaCl. The eluted preparation was dialyzed against 0.05 M Tris-HCl buffer (pH 7.5) and then applied to a second column containing 1 ml of DEAE-Sepharose CL-6B in order to concentrate the recombinant peptide. The gel was washed with 10 bed volumes of buffer, and recombinant peptide bound to the gel was eluted with 2 bed volumes of 0.05 M Tris-HCl buffer (pH 7.5) containing 0.15 M NaCl. The eluted preparation, containing 5.4 mg of protein per ml, was stored at 4°C.

Biological assays. Detection of cytotoxic, lethal, and enterotoxic activities and neutralization of these activities with specific toxin A antibody were done by previously described methods (7). In addition to Chinese hamster ovary K1 (CHO) cells, the mouse teratocarcinoma cell line OTF9-63 was used for cytotoxicity testing (16).

RESULTS

Construction of the toxin A gene. Three restriction fragments encompassing the *C. difficile* toxin A gene and a smaller ORF a short distance downstream from the toxin gene were ligated to produce the 9.4-kb construct in pCDtoxA.6. Exonuclease digestion removed all but 33 bases of the ORF located at the 3′ end of the toxin A gene, resulting in a clone designated pCDtoxA.03. Digestion with restriction endonucleases indicated that the gene fragments were properly inserted in the 8.6-kb insert of pCDtoxA.03 (data not shown). In addition, sequencing across the construct junction sites confirmed the correct ligation of the gene fragments.

Recombinant peptide expression levels were inadequate for our assays in the initial *E. coli* host strain χ1776. To obtain higher levels of expression, the recombinant plasmids were transferred to *E. coli* JM109, which grows more rapidly and to higher density than χ1776. Protein expression in JM109 with IPTG induction resulted in about a 10-fold increase in cytotoxicity compared with that produced without the addition of IPTG. Adequate levels of plasmid for sequencing and protein expression were difficult to obtain even in *E. coli* JM109, at least partially because of a high frequency of plasmid loss.

Cell lysates were tested for cytotoxicity on CHO and OTF9-63 cells. The latter cell line expresses a trisaccharide on its surface to which toxin A binds and is at least 100-fold more sensitive to toxin A than are CHO cells. The initial indication of toxic product expressed by pCDtoxA.6 in transformed *E. coli* χ1776 was observed on CHO cells as an elongation of cells with no cell rounding. The same lysate on OTF9-63 cells produced classic cell rounding, as seen with native toxin A, as well as spindle formation, at a final titer of 10^2. Lysate produced with IPTG induction from transformed *E. coli* JM109 produced cell rounding with both cell lines, although titers on CHO cells were low, never exceeding 10^2. Titers on OTF9-63 cells reached 10^3 to 10^4 with lysates produced from clones containing only the toxin gene or the toxin gene followed by the 695-bp ORF. This cytotoxic effect was neutralized by incubation of the lysates with goat affinity-purified toxin A antibody before application of the lysate to the cell culture. The excision of the ORF had no apparent effect on the level of cytotoxicity of the expressed protein.

Culture lysates of pCDtoxA.03 agglutinated rabbit eryth-

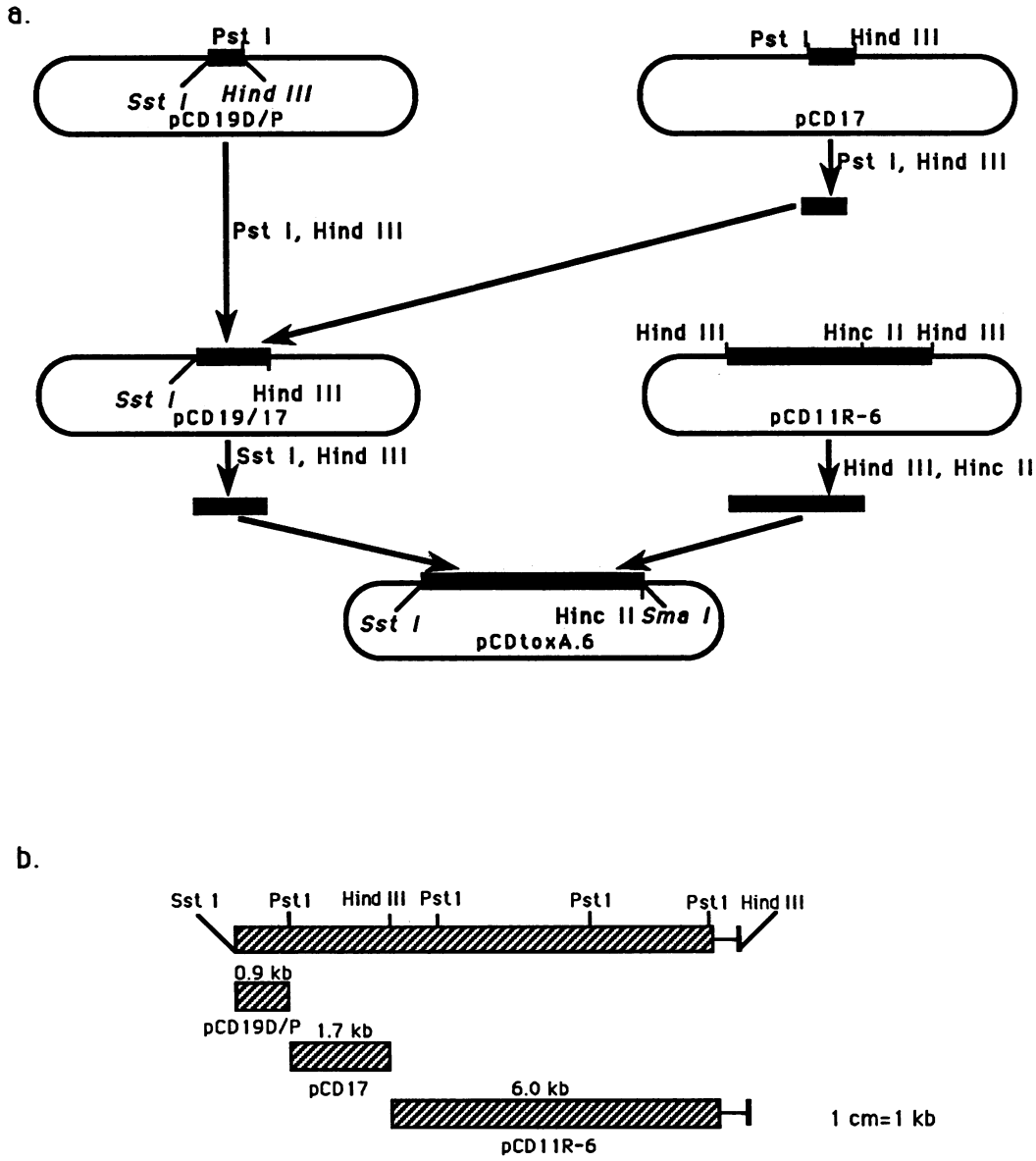

FIG. 1. (a) Strategy for construction of the intact toxin A gene of clone pCDtoxA.6. The 1.7-kb insert of pCD17 was ligated behind the 0.9-kb insert of pCD19D/P to create pCD19/17. The 6.8-kb *Hin*dIII-*Hin*cII fragment of the pCD11R-6 insert, as well as the insert of pCD19/17, was ligated into the *Sst*I-*Sma*I sites of pUC18 to create pCDtoxA.6. Thick lines are clostridial DNA inserts; thin lines represent the pUC plasmid. Vertical marks represent restriction endonuclease sites in insert DNA; diagonal lines represent restriction endonuclease sites in the vector multiple cloning region. (b) Partial restriction endonuclease map of pCDtoxA.03 and recombinant clone fragments used for construction of the intact toxin A gene. ⊣, 339 bases between the toxin A gene and the ORF as well as 33 bases of the ORF. Diagonal lines indicate restriction endonuclease sites in the vector multiple cloning region.

rocytes at a final titer of 1/16. *C. difficile* crude culture filtrate tested in the same assay produced hemagglutination at a final titer of 1/16. Lysates from clones containing only the pUC plasmid or the vector carrying the *C. difficile* toxin B gene did not cause hemagglutination. The presence of the ORF immediately following the toxin A gene had no effect on the hemagglutination titer of the toxic clones.

Purification of recombinant toxin A. Recombinant toxin A was purified from *E. coli* JM109 lysate by batch ion-exchange chromatography on DEAE-Sepharose CL-6B. The starting lysate (20 ml) had a protein concentration of approximately 10.6 mg/ml. The final purified material (4 ml) had a protein concentration of approximately 5.4 mg/ml. Analysis of the purified preparation showed that more than 90% of the protein was removed and that essentially all of the cytotoxic activity was recovered. Further analysis showed that the purified preparation was cytotoxic against CHO and OTF9-63 cells, with cytotoxic titers of 10^3 and 10^5, respectively. In addition, it was lethal when injected intraperitoneally into mice and gave a volume-to-length ratio of 0.7 ± 0.10 when assayed in the rabbit ileal loop assay. In each instance, the toxic activity was neutralized by specific toxin A antibody.

DISCUSSION

The gene coding for toxin A of *C. difficile* has been sequenced (4). None of the peptides encoded by the fragments used for sequencing of the gene were toxic on CHO cells, and no clones were obtained during the sequencing studies that encompassed the entire gene. However, we did obtain three clones containing inserts that together constituted the entire toxin A gene. Using these clones, we were able to reconstruct the entire toxin gene in a recombinant plasmid by ligation of adjacent fragments. Exonuclease digestion was then used to excise most of the extraneous clostridial DNA downstream from the toxin gene. Cloning of the intact gene without use of these preexisting cloned fragments would have been quite difficult because of the size of the gene and the lack of availability of unique flanking restriction enzyme sites not found within the gene itself. Knowledge of the exact sequence of each cloned fragment allowed us to clone the toxin gene free of other clostridial genes that might confuse the interpretation of peptide expression data.

Recombinant toxin A was readily expressed and purified from *E. coli* lysates and exhibited the cytotoxic, lethal, and enterotoxic activities observed with the native toxin purified from culture filtrates of *C. difficile*. The toxic peptide is expressed in *E. coli* JM109 with or without IPTG induction of the pUC vector's *lac* promoter, although at a diminished level without induction. A putative ribosome binding site (GGAGG) occurs a short distance before the translational start site of the toxin A gene (4); perhaps there is also a transcriptional start in the 70 bases preceding the toxin A gene within the cloned fragment. Another possibility is that the low level of expression in the absence of induction simply represents a basal level of activity from the *lac* promoter.

E. coli lysates containing the recombinant protein were cytotoxic to tissue culture cells, causing the typical rounding effect observed with native toxin A. Elongation of CHO cells, as seen in initial studies with lysates from recombinant *E. coli* χ1776, were also reported by Wren et al. (18). This cytotonic effect sometimes results from the application of extremely low levels of toxin A. Use of the more sensitive OTF9-63 cell line resulted in more classical cytotoxicity. The level of toxicity on OTF9-63 cells suggests that the amount of recombinant peptide in cell lysates is borderline for detection on CHO cells, which could explain the cytotonic effect. Purified, concentrated recombinant toxin caused complete rounding of both cell types.

Hemagglutination titers are much higher than would be expected from the level of cytotoxicity expressed by the toxic lysates. Native toxin A exhibiting the same level of hemagglutinating activity as does the recombinant peptide would have a concentration of about 10 μg/ml. This concentration level is not substantiated by the cytotoxicity levels of the recombinant toxin. Perhaps much of the recombinant peptide exists in an inactive form that retains the ability to bind rabbit erythrocytes but is not cytotoxic. In any case, inhibition of cytotoxicity with specific antibody indicates these activities are of toxin origin.

In this study, all biological activities of native *C. difficile* toxin—hemagglutination, cytotoxicity, enterotoxicity, and lethality—were elicited by a single polypeptide encoded by the 8.1-kb toxin A gene. The 695-bp ORF located at the 3′ end of the toxin A gene was unnecessary for the expression of toxin A activity. Preliminary data suggest that this ORF, as well as another at the 5′ end of the toxin A gene, is found exclusively in toxigenic *C. difficile*. The functions of these regions are now under investigation.

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