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Molecular cloning of *Clostridium difficile* toxin A gene fragment in λ gtll

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Toxin A of *Clostridium difficile* has been purified and monospecific antiserum produced. A reliable procedure for isolation and restriction of *C. difficile* chromosomal DNA was developed which allowed for the construction of a genomic library in λ gtll. Approx. 35000 plaques were screened using anti-toxin A which resulted in the identification of one stable positive clone, λ cd19. Verification of the immunological identity of the isolated toxin A gene fragment in λ cd19 was determined by affinity purifying toxin A antibodies specific for λ cd19 gene product, and using these selected antibodies to probe a Western blot of purified toxin A. The insert in λ cd19 was demonstrated to be a 0.3 kb fragment by restriction digestion, and by hybridization of the clone to a chromosomal digest of *C. difficile*. The peptide coded for by the toxin A gene fragment in λ cd19 was not cytotoxic for 3T3 mammalian tissue culture cells.

Enterotoxin; Antibiotic-associated colitis; λ gtll; DNA cloning

1. INTRODUCTION

Clostridium difficile is a toxin-producing strict anaerobic bacterium which is the causative agent for the diarrheal syndrome termed antibiotic associated pseudomembranous colitis [1,2]. Evidence exists which suggests that the pathogenic and cytotoxigenic effect of *C. difficile* is mediated by two toxins, A and B [3–6]. The enterotoxic activity of toxin A has been demonstrated to elicit a hemorrhagic fluid response in the rabbit intestinal loop assay, and causes fluid accumulation in the suckling mouse assay [4,5,7]. Toxins A and B have been shown to be cytotoxic for a variety of mammalian tissue culture cells [8,9]; however, toxin B which increases the ratio of globular to filamentous actin [10] is consequently more cytotoxic than toxin A.

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Toxin A has been purified and partially characterized [3,6,8]. This enterotoxin is a heat-labile protein which has been reported to have an M_r of approx. 500000 [3]; however, the subunit composition of the protein has not been clearly elucidated. Toxin A has been determined by amino acid analysis [6,7,11] to have a high degree of hydrophobic amino acids and to share some similarities with the composition of toxin B [6].

To obtain information on the structure of toxin A, and to study the regulation of toxin production in different strains of *C. difficile*, the toxin gene must be cloned and characterized. The initiation of this investigation is reported herein. We have identified a DNA fragment of the toxin A gene of *C. difficile* using an antibody probe.

2. MATERIALS AND METHODS

2.1. *Purification of toxin A*

C. difficile toxin A was purified from strain

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10463 (supplied by Tracy D. Wilkins, Virginia Polytechnic Institute, Blacksburg, VA), by CL-6B DEAE-Sepharose column chromatography and acetic acid precipitation as described by Sullivan et al. [3] with modifications. Fractions collected after column chromatography that contained toxin A and several contaminating proteins were not discarded. These fractions were pooled, acetic acid precipitated at pH 5.5, and loaded on a 1.5 × 15 cm column of CL-6B DEAE-Sepharose which had been equilibrated with 50 mM Tris-HCl (pH 7.5) containing 0.05 M NaCl. The column was washed with 100 ml of the same buffer and eluted with a 200 ml linear 0.05–0.25 M NaCl gradient in 50 mM Tris-HCl (pH 7.5). Toxin activity was monitored by the cytotoxicity assay using 3T3 mouse fibroblasts.

2.2. *Anti-toxin A preparation*

Antiserum was produced in a 5 kg New Zealand White rabbit against formalin-treated toxin A utilizing the procedure of Ehrlich et al. [12]. Anti-toxin used for screening recombinant clones was adsorbed with *E. coli* Y1090 and λgt11 to remove interfering antibodies.

2.3. *Isolation of chromosomal DNA*

Isolation of high-M_r chromosomal DNA from *C. difficile* was accomplished by growing strain 10463 overnight in 1 l of brain heart infusion media containing 10 mg/ml lysozyme. The pelleted cells were washed in TSE buffer (25 mM Tris, 10% sucrose, 0.1 M EDTA, pH 8.0), and resuspended in 40 ml TSE with an increased EDTA concentration of 0.25 M. Next, 4 ml lysozyme (35 mg/ml) was added and incubated at 25°C for 1 h. An equal volume of 33% SDS in 0.25 M EDTA (pH 8) was added, mixed gently for 5–20 s and extracted immediately with an equal volume of phenol/chloroform. Four additional phenol/chloroform extractions and one chloroform/isoamyl extraction was performed followed by ethanol precipitation [13].

2.4. *Construction and screening of* C. difficile *genomic library*

Fragments of *C. difficile*'s toxin A gene were cloned and screened using the λgt11 expression system of Young and Davis [14] with the following alterations. *C. difficile* DNA was partially digested with *Taq*I (approx. 1–10 kb), *Eco*RI methylase treated and ligated to three different size *Cla*I-*Eco*RI adapters (kindly provided by Ken Stover, Walter Reed Army Research Institute, Washington, DC, unpublished). Excess adapters were removed by digestion and polyethylene glycol fractionation [15]. After ligation into the unique *Eco*RI restriction site of λgt11 and packaging of the recombinant DNA (Packagene, Promega Biotec, Madison, WI), the library was directly screened without amplification of the phage particles. Toxin A antigen-producing plaques were visualized on nitrocellulose filters using a 1:200 dilution of *E. coli*-λgt11 adsorbed anti-toxin A, and the Bio-Rad horseradish peroxidase-bound goat anti-rabbit IgG immunoblot kit (Richmond, CA).

2.5. *Antigen-selected antibodies*

Antibodies specific for the toxin-positive peptides encoded by recombinant λgt11 phage particles were affinity purified from toxin A antiserum using the procedure of Lyon et al. [16] with the following variations. Recombinant phage were plated at a density of approx. 6000 plaques per plate (150 mm diameter). Nitrocellulose filters were overlaid on the agar plates and incubated at 37°C for 16 h which allowed for binding of antigens. The filters were next removed and suspended overnight in a 1:50 dilution of *E. coli* adsorbed toxin A antiserum. After washing the nitrocellulose paper twice in TTBS (0.05% Tween 20, 50 mM Tris, 150 mM NaCl, pH 8.0) and once in 10 ml saline, specific antibodies which bound to the nitrocellulose filters were eluted by adding 10 ml glycine buffer per filter (0.2 M glycine, 0.15 M NaCl, pH 2.8) for 10 min. Next the nitrocellulose paper was removed from the glycine buffer and the pH of this selected antibody solution was neutralized by adding 8 mg Tris per ml eluate.

2.6. *Electrophoresis and blotting*

Non-denaturing polyacrylamide gel electrophoresis (PAGE) of toxin A was performed in a 5–15% gradient slab gel at pH 8.3 [17]. Gels were stained with Coomassie blue R-259. Electrophoretic transfer (Western blotting) of proteins from polyacrylamide gels to nitrocellulose paper was performed as described by Towbin et al. [18].

DNA was separated on a 0.8% agarose gel and stained with ethidium bromide [13]. Nick translation with [α-^{32}P]dCTP and Southern blot hybridizations were performed as described in the nick translation and sure blot hybridization kits provided by Oncor (Gaithersburg, MD).

2.7. *Cytotoxicity testing*

Supernatant from *C. difficile* cultures, purified or partially purified toxin A, and crude lysates from *E. coli* Y1090 (obtained from Promega Biotec) infected with recombinant λgt11 [13] and induced with isopropyl thio-β-D-galactopyranoside (IPTG) [14], were filter sterilized and cytotoxic activities determined using 3T3 mouse fibroblasts as in [19].

3. RESULTS AND DISCUSSION

3.1. *Isolation of toxin A and production of anti-toxin*

After modifying the toxin purification procedure described by Sullivan et al. [3], the total yield of purified toxin A was increased by 20%. This was accomplished by pooling fractions from the first DEAE-Sepharose column that contained toxin A and several contaminating proteins, acetic acid precipitating and running these fractions twice on DEAE-Sepharose columns (table 1). The cytotoxic activity of toxin A is 1000-fold lower than the activity of toxin B [3]; therefore, the minimal toxic dose of toxin A on 3T3 tissue culture cells could not be determined until after DEAE-Sepharose chromatography. The purity of the active toxin preparation was determined by polyacrylamide gel electrophoresis (fig.1, lane 1), and immunoelectrophoresis using crude culture supernatant antiserum purchased from Virginia Polytechnical Institute [12] (not shown). Antiserum against toxin A was generated as described in section 2 (fig.1, lane 2).

3.2. *Cloning and characterization of toxin A*

The harsh conditions needed to lyse *C. difficile* and the high nuclease activity associated with this bacterium prevented the use of standard procedures for isolation of high-M_r chromosomal DNA. A purification procedure has been developed and described in section 2 which yields high-M_r *C. difficile* chromosomal DNA (>50 kb)

Table 1

Purification of *C. difficile* toxin A

Stage	Purification step	Volume (ml)	Total protein (mg)	Minimal[a] toxic dose (ng)	Purification (-fold)
1	Crude culture supernatant	65	84.5		
2	First purification[b]				
	CL-6B DEAE	32	5.8	36	1
	Acetic acid precipitation	3.2	1.0	6.4	5.6
3	Second purification[c]				
	CL-6B DEAE	20	8.9	8.9	1
	Acetic acid precipitation	3.2	3.0	1.9	4.6
	CL-6B DEAE	3.0	0.2	1.3	6.8

[a] Minimal toxic dose delineates the estimated amounts of protein/well after serial dilution which caused 50% of rounding of 3T3 fibroblasts

[b] Purification procedure described by Sullivan et al. [3]

[c] Second purification represents isolation of toxin A from fractions in the DEAE column in stage 2 with 3–5 contaminating proteins

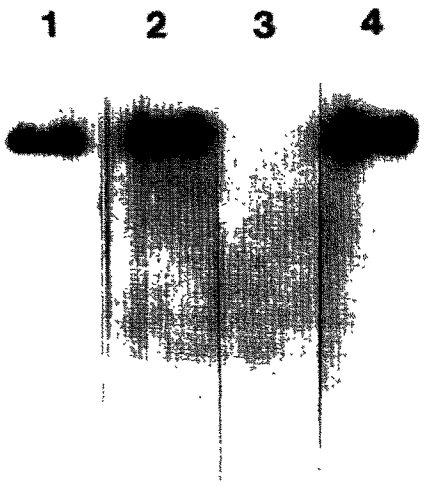

Fig.1. Purity of toxin A, and confirmation of cloned toxin determinants. PAGE and Western blot analysis were performed as described in section 2. Lanes: (1) PAGE of 6 μg purified toxin A; (2) Western blot of toxin A using control toxin A antiserum, 1:200 dilution; (3) Western blot of toxin A with selected antibodies from λgt11 control plaques; (4) Western blot of toxin A with antibodies selected from λcd19.

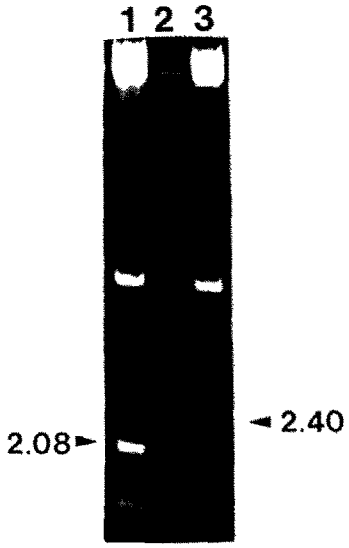

Fig.2. Restriction digestion of λgt11 and λcd19. λgt11 (lane 1) and λcd19 (lane 3) were double digested with *Sac*I and *Kpn*I as described by the supplier. The band immediately above the 2.4 kb fragment in lane 3 represents incomplete digestion of the 2.4 kb fragment and the lower migrating 1.51 kb fragment. Lane 2 contains the M_r standard *Hin*dIII-digested λ DNA (BRL Inc., Gaithersburg, MD).

that can be digested with selective restriction enzymes.

A genomic library of *C. difficile* DNA was constructed in λgt11. Approx. 35000 plaques were screened for toxin A antigen-producing clones resulting in the original detection of 22 positive plaques. All but one of the identified positive plaques spontaneously lost the *C. difficile* inserts after plaque purification and amplification as indicated by immunologically testing for toxin A cloned antigens, and by reversion from clear to blue plaques. λcd19 was the only recombinant plaque stable enough to allow for consistent immunological verification; however, this clone reverts from clear to blue plaques at a frequency of 2%. Verification of the immunological identity of the isolated toxin A gene fragment in λcd19 was determined by affinity purifying toxin A antibodies specific for λcd19 gene product, and using these selected antibodies to probe a Western blot of purified toxin A (fig.1).

The *Eco*RI cloning site in λgt11 is contained within a 2.08 kb *Sac*I-*Kpn*I fragment [14]. In the hybrid clone λcd19, this *Sac*I-*Kpn*I fragment was increased in size from 2.08 to 2.40 kb, thereby

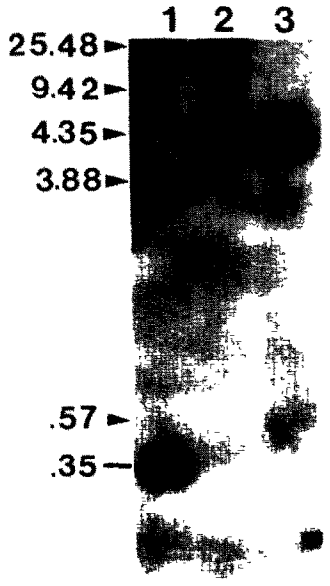

Fig.3. Southern hybridization of restriction digested *C. difficile* chromosomal DNA with [α-^{32}P]dCTP-labeled λcd19. *C. difficile* chromosomal DNA was digested with *Taq*I (lane 1), *Hin*dIII (lane 2) and *Pst*I (lane 3).

demonstrating the presence of a 0.3 kb *Taq*I insert (fig.2). (N.B., the genomic library was generated with *Taq*I.) A 0.3 kb *C. difficile* DNA insert in λcd19 was also demonstrated by hybridizing (α-^{32}P)-labeled nick-translated λcd19 DNA to a *C. difficile Taq*I chromosomal digest (fig.3). The (α-^{32}P)-labeled cd19 probe also hybridized to a 4.5 kb fragment in a *Pst*I chromosomal digestion of *C. difficile*, and a 16 kb fragment in a *Hin*dIII digestion (fig.3).

The recombinant λcd19 gene product is immunologically positive for anti-toxin A, but does not code for the active toxin A protein. *E. coli* lysates generated from infecting Y1090 with λcd19 phage particles and induced with IPTG were negative for cytotoxic activity on 3T3 mammalian tissue culture cells.

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