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Molecular cloning and expression of *Clostridium difficile* toxin A in *Escherichia coli* K12

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Clostridium difficile toxin A was purified to homogeneity and was used to raise monospecific antiserum in rabbits. A gene bank of *C. difficile* DNA in *Escherichia coli* was constructed by cloning *Sau*3A-cleaved clostridial DNA fragments into the bacteriophage vector λEMBL3. Out of 4500 plaques screened with antitoxin A, 9 clones were positively identified. One of these clones λtA5 expressed a 235 kDa protein which exhibited a cytotonic effect on Chinese hamster ovary cells, and had the ability to haemagglutinate rabbit erythrocytes, both properties characteristic of toxin A. The size of the λtA5 insert DNA was 14.3 kb.

Toxin A; Molecular cloning; Pseudomembranous colitis; *(Clostridium difficile)*

1. INTRODUCTION

Clostridium difficile has been shown to be the causative agent of pseudomembranous colitis and antibiotic-associated colitis and diarrhoea in humans and animals [1–4]. The pathogenicity of this organism has been linked to its toxin producing capabilities [1,3]. It appears to elaborate at least two toxins; toxin A which is an enterotoxin capable of inducing fluid accumulation in the rabbit ileal loop assay, and toxin B which is a potent cytotoxin [5,6]. Toxin A also exhibits cytotoxic activities [3,6] and had a cytotonic effect on Chinese hamster ovary (CHO) cells [7]. Both toxins also have the ability to haemagglutinate rabbit erythrocytes [8,9].

Much controversy exists concerning the biochemical composition of toxin A such as amino acid content [10–12], isoelectric points [10,13] and relative molecular mass (estimates range from 57 to 600 kDa [6,7,12–16]). The molecular cloning of toxin A will provide more definitive information on the polypeptide's structure and mode of action. Furthermore, highly purified toxin A and antitoxin A sera are necessary in the development of reliable detection systems for toxigenic strains of *C. difficile*.

Recently, Muldrow et al. [17] have reported the cloning of a toxin A gene fragment in λgt11, but no biological activity of the toxin was observed. In our study *C. difficile* toxin A was cloned and expressed in *E. coli* K12, using the bacteriophage replacement cloning vector λEMBL3.

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2. MATERIALS AND METHODS

2.1. *Bacterial strains*

An isolate of *C. difficile* (W1) obtained from a patient with pseudomembranous colitis at this hospital was used for the purification of toxin A and the construction of a gene library of *C. difficile* DNA. The identity of the strain was confirmed by smell, morphology, fluorescence under UV and gas-liquid chromatographic analysis of volatile fatty acids. *E. coli* K12 392 (lysogenic for

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phage P2) was used as the host strain for cloning manipulations [18].

2.2. *Purification of toxin A*

A 5 l growth of *C. difficile* was cultured as described by Redmond et al. [19]. Toxin A was purified from the culture filtrate by 40% ammonium sulphate precipitation, preparative electrophoresis using a discontinuous buffer system and by ion-exchange chromatography on a DEAE-Sepharose CL-6B column [19]. The purified toxin A produced a single band on SDS-PAGE analysis and caused significant fluid accumulation in the rabbit ileal loop assay [19].

2.3. *Antitoxin A preparation*

Antisera to toxin A was raised in 3 kg male Californian rabbits by the procedure of Redmond et al. [19]. Antitoxin A used for screening recombinant clones was adsorbed at least three times with whole cell and λEMBL3 lysed *E. coli* cells to remove non-specific antibodies.

2.4. *Construction of* C. difficile *gene library*

C. difficile genomic DNA was prepared by a method developed in this laboratory [20]. *C. difficile* DNA was partially digested with *Sau*3A (producing fragments of approx. 9–20 kb in size) and ligated into *Bam*HI-digested λEMBL3 DNA. After ligation, the gene library was packaged (Gigapack kit, NBL, Cramlington, England) and screened directly without amplification.

2.5. *Screening of* C. difficile *gene library*

The *C. difficile* gene library was screened for toxin A positive recombinants by a method similar to that described by Russell et al. [21]. The gene library was plated on *E. coli* K12,392 to give approx. 300 plaques per 90 mm diameter plate. Gridded nitrocellulose filter discs (Schleicher and Schuell, England) were overlaid on agar plates and left in contact at 4°C for 1 h to adsorb antigenic products from the recombinant clones. The nitrocellulose was removed from the agar and excess sites were blocked with 3% bovine serum albumin (BSA) in buffered saline (0.9% NaCl, 10 mM Tris; pH 7.4). The nitrocellulose was incubated at 25°C for 2 h with *E. coli* adsorbed toxin A antiserum, diluted 1:100 in 3% BSA solution. The incubation with the anti-rabbit IgG and visualisation of the immunoreactive protein product were performed as described in [22]. The position of the plaques which reacted immunogenically with antitoxin A were identified on the original agar plates. The positive plaques were picked and stored in 0.5 ml SM buffer (0.1 M NaCl, 10 mM $MgSO_4$, 50 mM Tris (pH 7.5), 0.01% gelatine) and 20 μl chloroform. Samples for immunoblot analysis and biological assays were obtained from single plaques after plating each with *E. coli* to produce a lawn of confluent lysis. Lawns were overlaid with SM buffer (0.25 ml) for 1 h at 25°C and harvested.

2.6. *SDS-PAGE and immunoblotting*

SDS-PAGE, electrophoretic transfer (immunoblotting) and the development of immunoreactive protein products was performed as described in [22].

2.7. *CHO cells assay and neutralisation tests*

Morphological changes of CHO cells were examined essentially as described by Katoh et al. [7].

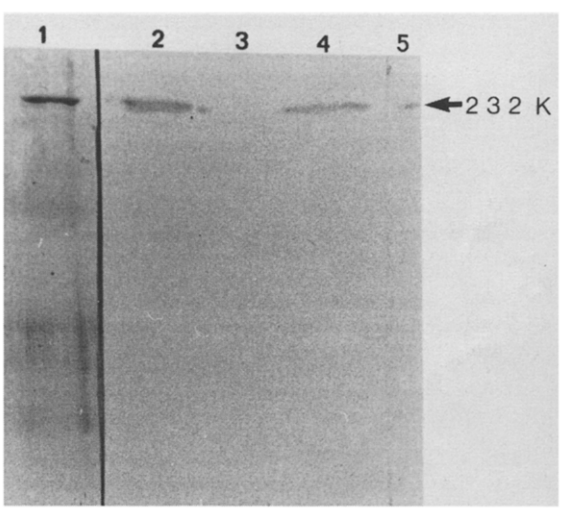

Fig.1. Confirmation of cloned toxin A determinant by immunoblotting as described in section 2.6. Lanes: (1) 20 μl of λtA5 plaque lysate against *E. coli* adsorbed antitoxin A provided by P. Rautenberg [13]; (2) purified toxin A (4 μg) against antitoxin A prepared as described in section 2.3; (3) 20 μl of λEMBL3 control plaque lysate against antitoxin A as lane (2); (4) 20 μl of λtA5 plaque lysate against antitoxin A as lane (2); (5) molecular mass marker (7.5 μg) catalase (232 kDa).

Samples for assay were double diluted in the tissue culture test medium and 20 μl volumes of each dilution were added to the wells. For neutralisation tests, mixtures containing toxin A were incubated with doubling dilutions of antitoxin A or *C. sordellii* antitoxin (Wellcome Biotechnology, England) for 1 h at 37°C prior to addition to CHO cells.

2.8. *Haemagglutination assay*

Samples (20 μl) were tested for their ability to haemagglutinate rabbit erythrocytes (Sigma, England) at 4°C and 37°C by the method described by Franzon and Manning [23].

2.9. *Isolation of bacteriophage λEMBL3 DNA*

The plate lysate method [24] was used for the isolation of DNA from recombinant λEMBL3 clones. Restriction endonuclease digestion of recombinant λEMBL3 DNA was carried out as recommended by the manufacturers (NBL, Cramlington, England).

3. RESULTS AND DISCUSSION

A total of 4500 λEMBL3 plaques were screened for toxin A antigen-producing recombinants, of which 9 reacted positively. One of these clones named λtA5 was chosen for further study. Extraction of DNA from λtA5 and digestion with *Bam*HI or *Sal*I revealed a DNA insert of 14.3 kb. Immunoblot analysis with λtA5 showed a single protein band of M_r 235000 reacting antigenically with *E. coli* adsorbed antitoxin A (fig.1, lane 4), which corresponded to the same relative position for the

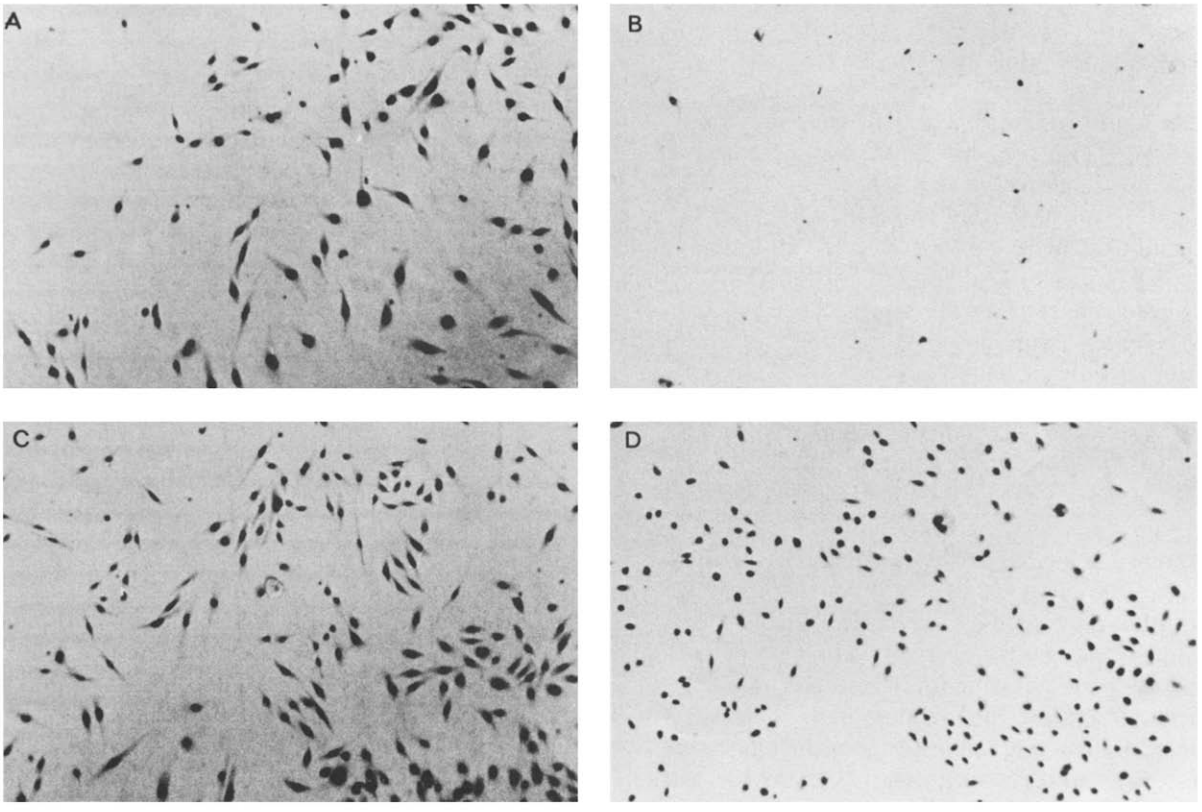

Fig.2. Photomicrographs of CHO cells stained with Giemsa: A, cells treated with 1 μg/ml of toxin A; B, cells treated with 8 μg/ml of toxin A; C, cells treated with 20 μl of λtA5 plaque lysate; D, cells treated with 20 μl of λEMBL3 control plaque lysate. The neutralising effect of antitoxin A and *C. sordellii* antitoxin on toxin A and λtA5 plaque lysate resulted in the normal appearance of the cells, as in D.

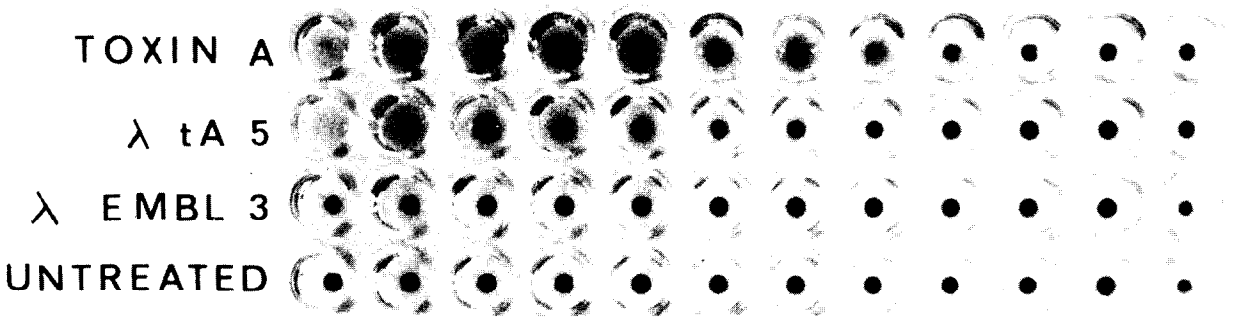

Fig.3. Haemagglutination of rabbit erythrocytes at 4°C. The wells contain two-fold serial dilutions of toxin A 8 μg/ml (first row), 20 μl of λtA5 plaque lysate (second row), 20 μl of λEMBL3 control plaque lysate (third row), and untreated rabbit erythrocytes (fourth row). The effects of antitoxin A and *C. sordellii* antitoxin on toxin A and λtA5 plaque lysate were the same as rows 3 and 4.

purified toxin A (fig.1, lane 2). No protein bands were seen for the control λEMBL3 plaque lysate (fig.1, lane 3). Rautenberg and Stender [13] have reported toxin A as having an M_r value of 230000 when tested by SDS-PAGE, which corresponds closely to the protein product of λtA5 of M_r 235000. Furthermore, antitoxin A, kindly provided by P. Rautenberg, cross-reacts with λtA5 in the 230–235 kDa region (fig.1, lane 1).

Incubation of toxin A (1 μg/ml) and λtA5 lysate (20 μl) with CHO cells caused them to elongate (fig.2A,C). This cytotonic effect was not observed using the control λEMBL3 plaque lysates (fig.2D), and could be completely neutralised for purified and cloned toxin A using antitoxin A or *C. sordellii* antitoxin where the CHO cells appeared normal. At higher concentrations of toxin A (>5 μg/ml) a cytotoxic effect was observed where the toxin destroys the CHO cells and the cytotonic effect is masked (fig.2B). Using 100 μl of λtA5 rounding up of cells was partially visible (unpublished). Katoh et al. [7] have also reported a cytotonic effect using low concentrations of toxin A (0.9–3.6 μg/ml) and similar effects on CHO cells have been shown to be induced by cholera enterotoxin [25] and enterotoxigenic *E. coli* heat-labile enterotoxin [26].

Purified toxin A (5 μg/ml) and λtA5 lysate (20 μl) caused significant haemagglutination when incubated with rabbit erythrocytes at 4°C (fig.3). This effect was diminished when the microtitre plates were incubated at 37°C and no effect was seen with the control plaque λEMBL3 at 4°C or 37°C. The haemagglutinating effect of purified toxin A and λtA5 lysate could be inhibited by incubating with antitoxin A or *C. sordellii* antitoxin for 1 h prior to the addition of rabbit erythrocytes (not shown). Krivan et al. [8] have reported the ability of toxin A to bind avidly to rabbit erythrocytes, particularly at 4°C, and have proposed that the binding site for toxin A in the gut may be similar to outer membrane carbohydrate moieties from rabbit erythrocytes.

A previous report [17] concerning the cloning of toxin A demonstrated that a clone, designated λCd25, carrying a 0.3 kb DNA fragment, encoded a protein which reacted antigenically with antitoxin A on immunoblotting, although no molecular mass estimate for the protein was given [17]. The authors' clone, λCd25, showed no toxic effects on tissue culture cells, in contrast to λtA5 described in this paper. This may be related to the fact that λtA5 has a larger DNA insert than λCd25, 14.3 kb compared with 0.3 kb, and is therefore more likely to contain the genetic information necessary for the expression of toxin A. This study has demonstrated that *C. difficile* toxin A can be conveniently cloned and expressed in *E. coli* using the λ bacteriophage vector EMBL3.

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