Cloning and Characterization of Overlapping DNA Fragments of the Toxin A Gene of *Clostridium difficile*

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Clostridium difficile, a human pathogen, produces two very large protein toxins, A and B (250–600 kDa), which resist dissociation into subunits. To clone the toxin A gene, a genomic library of 3–8 kb chromosomal DNA fragments of *C. difficile* strain VPI 10463 established in pUC12 was screened with a rabbit polyclonal toxin A antiserum. Thirty-five clones were isolated which carried 2.5–7.0 kb inserts representing a 10 kb region of the *C. difficile* genome. All the inserts were oriented in the same direction, suggesting that toxin A gene expression was under control of the *lac* promoter of the pUC12 vector. Western blot experiments revealed the presence of low amounts of fusion proteins of variable size (30–170 kDa) in *Escherichia coli* strains harbouring recombinant plasmids. As deduced from subcloning experiments, the DNA sequences encoding toxin A comprised about 4 kb, corresponding to about 140 kDa of the 300–600 kDa protein. This was either due to incomplete cloning of the gene or it might indicate a subunit composition of toxin A. No additional gene(s) with homology to the cloned toxin A gene was detected.

INTRODUCTION

Many *Clostridium* species produce toxins as factors of pathogenicity. For some of the most prominent representatives, like *Clostridium tetani*, *Clostridium botulinum* and *Clostridium perfringens*, intensive studies have been undertaken to characterize the toxins at the protein level (Middlebrook & Dorland, 1984). Toxinogenic *Clostridium difficile* strains are correlated with a pseudomembranous colitis (PMC) (Bartlett *et al.*, 1978). The development of this disease, which may even be lethal, is a severe side effect of treatment of patients with various antimicrobial agents (George, 1986). The disease is correlated with the presence of toxins whose role in the progress of the PMC is not yet exactly defined. Two very large toxins, A and B (Sullivan *et al.*, 1982) or D₁ and D₂ (Banno *et al.*, 1984), have been described, with molecular masses of 300–600 kDa (A), and 250–500 kDa (B) and, additionally, two low-molecular-mass proteins also called A and B (52 kDa toxin A, Rihn *et al.*, 1984; 150 kDa toxin B, Pothoulakis *et al.*, 1986). The amino acid composition of the high-molecular-mass toxins is similar (Lyerly *et al.*, 1986b). Their dissociation into subunits by mercaptoethanol treatment has not been achieved. At present the molecular masses of toxins A and B are not precisely known. Despite their mercaptoethanol resistance, the possibility still remains that they comprise a number of subunits, as was deduced from the reactivity of toxin A with monoclonal antibodies (Lyerly *et al.*, 1986a).

Immunological characterization of the two toxins has led to conflicting results. Polyclonal antibodies raised against A and B react specifically with the toxin used for immunization and neutralize specifically the activity of the homologous toxin (Laughon *et al.*, 1984). However, some monoclonal antibodies (mAbs) cross-react with both toxins regardless of the toxin used for

Abbreviation: mAb, monoclonal antibody.

immunization (v. Eichel-Streiber *et al.*, 1987; Lyerly *et al.*, 1986a). These results are further complicated by the finding that polyclonal *Clostridium sordellii* antitoxin neutralizes the activity of both toxin A and toxin B (Chang *et al.*, 1986).

The activities of the two toxins *in vivo* and *in vitro* appear to differ. When added to cultures of CHO cells, toxin B is about 1000-fold more active than toxin A. Injected intraperitoneally, both toxins have about the same toxicity (Sullivan *et al.*, 1982; v. Eichel-Streiber *et al.*, 1987). After gastric application, only toxin A exerts a toxic effect (Lyerly *et al.*, 1985a). These results, taken together with the reactivity of toxin A and B towards mAbs (Lyerly *et al.*, 1985b; Lyerly *et al.*, 1986a), have led us to postulate that toxin A may represent a precursor form which is processed to toxin B after transport and specific interaction with a target cell (v. Eichel-Streiber *et al.*, 1987).

Recombinant DNA techniques have previously been used to show homologies at the genetic level between toxins isolated from different bacteria (Timmis *et al.*, 1984). However, little is known about the genetics of these highly toxic clostridial toxins. The tetanus toxin gene has been cloned and sequenced (Eisel *et al.*, 1986; Fairweather *et al.*, 1986). The cloning of a 0.3 kb fragment of the *C. difficile* toxin A gene has also been reported recently (Muldrow *et al.*, 1987). We here present the cloning and molecular characterization of a 10 kb region encoding part, at least, of toxin A. In addition, we have tested whether a single gene encodes both toxins A and B.

METHODS

Strains, enzymes, and chemicals. The toxinogenic *C. difficile* strain VPI 10463 was kindly supplied by N. Sullivan (Sullivan *et al.*, 1982) and grown in brain/heart infusion broth (Bio Merieux, France) at 39 °C under anaerobic conditions without shaking. For cloning experiments *Escherichia coli* was grown in LB medium (Maniatis *et al.*, 1982), with vigorous shaking at 37 °C for JM83 and JM101 (Yanisch-Perron *et al.*, 1985), and at 30 °C for pop2136 – this strain carries the cI857(Ts) repressor (Haymerle *et al.*, 1986). Enzymes were supplied by Boehringer and BRL, chemicals by Sigma and Merck.

Antibody production. Two-month-old New Zealand White rabbits were immunized intramuscularly with FPLC-purified toxin A (v. Eichel-Streiber *et al.*, 1987). For the first injection a 1:1 mixture of 200 µl protein (10 µg) and 200 µl complete Freund's adjuvant was used. Further booster injections were given at 10–14 d intervals using toxin and incomplete Freund's adjuvant (200 µl each). The first challenge was carried out with 10 µg toxin, and five additional injections with 20 µg of toxin. Serum was taken and analysed by ELISA and immunoblotting as previously described (v. Eichel-Streiber *et al.*, 1987).

Isolation of DNA, restriction analysis and cloning. *C. difficile* was grown to the early exponential phase to isolate chromosomal DNA. The DNA was prepared using methods described by Miura (1967). Partial digestion of the prepared DNA, and restriction analysis and subsequent cloning of sized DNA, were done by established procedures (Maniatis *et al.*, 1982). DNA minipreparations were done according to the procedure of Volkaert (1987).

The expression vectors pEX1, 2 and 3 were used for subcloning experiments (Stanley & Luzio, 1984). These vectors allow fragments to be cloned in all three reading frames. Expression is under the control of the cI857(Ts) repressor (chromosomally encoded by the recipient *E. coli* strain pop2136) and may be induced by shifting the growth temperature in the early exponential phase from 30 °C to 42 °C.

Construction of a genomic library. Recombinant DNA techniques were used according to the guidelines of the Bundesministerium für Forschung und Technologie, FRG. *C. difficile* DNA (fragments larger than 50 kb) was digested with a mixture of *AluI* and *HaeIII* into fragments of 1–20 kb in length. Fragments in the size range 3–8 kb (2.0 µg sample), isolated from agarose gels (Maniatis *et al.*, 1982), were ligated to 0.25 µg *SmaI*-digested and dephosphorylated pUC12 (Messing, 1979). The recombinant DNA was introduced into *E. coli* JM83 by transformation (Hanahan, 1983). Cells were plated out on 24 × 24 cm plates. On six plates 70 000 independent clones were obtained. They were resuspended in LB containing ampicillin (100 µg ml⁻¹), supplemented with 10% (v/v) glycerol, divided into 50 µl portions and frozen at –70 °C. An 800 µl volume of a 1:10 000 dilution of the frozen stock solution was plated to achieve a cell density of 12 000 on a 24 × 24 cm plate.

Isolation of toxin-A-positive clones. The 70 000 colonies plated out on six 24 × 24 cm plates (see above) were screened using a procedure described by Stanley & Luzio (1984). For colony blotting, rabbit toxin A antiserum was used at a dilution of 1:2000. Anti rabbit-peroxidase conjugate (Dako) was taken at a dilution of 1:1000. Staining was done with diaminobenzidine (0.5 mg ml⁻¹) as substrate in the presence of hydrogen peroxide (0.06 µg ml⁻¹). Positive clones were isolated as single colonies and retested for toxin expression. Recombinant clones were also tested for toxin activity in the CHO test as described previously (v. Eichel-Streiber *et al.*, 1987).

Subcloning of pCd insert DNA. A 1.9 kb *PstI* fragment was isolated from pCd14. It extends from the *PstI* site at

2.6 kb to the *Pst*I site in the polylinker at the right side of the insert DNA (see Fig. 2). This fragment was cloned into *Pst*I-digested and dephosphorylated pEX1, 2 and 3 in both orientations.

Hybridization experiments. These were done by standard procedures (Southern, 1975; Reed & Mann, 1985). In order to detect toxin-A-related genes within the genome of *C. difficile* VPI 10463, 4 µg chromosomal DNA was digested for 3 h with 40 units of the appropriate restriction enzymes and hybridized with pCd10 or pCd14 insert DNA. Radioactive DNA probes were labelled with [α -³²P]dATP (Amersham) by nick translation (Rigby *et al.*, 1977). After hybridization, washing was carried out at various stringencies equivalent to between 13 °C and 47 °C below the melting point (T_m) of the DNA (T_m : 84 °C, measured in 5 × SSC solution). The conditions equivalent to 13 °C below T_m were 57 °C, 50% (v/v) formamide, 0.1 × SSC; those equivalent to 47 °C below T_m were 23 °C, 0% formamide, 0.1 × SSC (Howley *et al.*, 1979). (1 × SSC is 0.15 M-NaCl, 0.015 M-trisodium citrate, pH 7.0).

Recombinant protein. A fresh 5 ml LB + ampicillin culture was inoculated with 50 µl of recombinant clones grown overnight. After 3–4 h, cells were harvested by centrifugation, and resuspended in 250 µl sodium phosphate buffer (25 mM, pH 7.5) and 250 µl SDS-PAGE dye solution without 2-mercaptoethanol (Laemmli, 1970). After 10 min incubation at 95 °C the lysed cell suspension was sonicated for 5–10 s to reduce the viscosity. The pEX subclones were grown to early exponential phase at 30 °C and then shifted to 42 °C by adding an equal volume of growth medium heated to 54 °C. After this induction, cells were grown for a further 1 h. Total cell lysates were prepared as described above. Gel electrophoresis and Western blotting were done as previously described (v. Eichel-Streiber *et al.*, 1987). Three antibodies were used sequentially for staining: toxin A antiserum (1:2000 dilution), goat anti-rabbit serum (1:1000 dilution; Dianova), and rabbit anti-goat-peroxidase conjugate (1:1000 dilution; Dako). In all three antibody incubations *E. coli* absorption protein was included (1:10 v/v) to reduce the background generated by *E. coli*-specific antibodies present in almost every polyclonal serum. Absorption protein was prepared from a 5 litre culture of *E. coli* JM83 in the late exponential phase. Cells were harvested, resuspended to 1% of their original volume in Tris/HCl (25 mM, pH 8.0) plus sucrose (12.5% w/v) and digested with lysozyme (25 ng ml⁻¹) for 40 min. Triton X-100 (0.1%) was added to give 3% of the original volume, then the lysate was heated to 65 °C for 10 min, centrifuged for 30 min at 25000 r.p.m. (Beckman, 45 Ti rotor) and the supernatant used as absorption protein.

In Western blot experiments with mAbs, a 1:1000 dilution of the chosen antibodies [mAbs 1339, 1134 and 1337; rabbit anti-mouse-peroxidase conjugate (Dako)] was used. For screening of the genetic library, a mixture of four mAbs was used (mAbs 1339, 1322, 1142 and 1134; v. Eichel-Streiber *et al.*, 1987). The isolated recombinant proteins were stained with a 1:2 dilution of cell supernatant of hybridoma cell line 1337.

RESULTS AND DISCUSSION

Construction of a genomic library of C. difficile and screening with toxin A antiserum

In order to clone the toxin A gene, a genomic library of randomly cut chromosomal DNA of *C. difficile* VPI 10463 was initially established. Toxins A and B of VPI 10463 have been reported to have molecular masses between 150 and 500 kDa (depending on the experimental conditions). Genes encoding proteins of this size would occupy 7–16 kb of DNA. To avoid the expression of the complete, functional toxin A (600 kDa on native PAGE gels, 300 kDa on SDS-PAGE for *C. difficile* strain VPI 10463) and thus follow the safety guidelines, an overlapping set of fragments smaller than 8 kb was cloned. Partial digestion with *Sau*3A could not be used successfully to generate a continuous smear of fragmented DNA on agarose gels. However, using *Alu*I and *Hae*III together, fragments of different size ranges could be produced depending on the conditions used.

DNA cut with *Alu*I/*Hae*III was separated on an agarose gel and fragments of 3–8 kb were isolated and ligated with the *Sma*I-cut and dephosphorylated vector pUC12 (Messing, 1979). In pUC12, a representative library of 70000 independent clones was obtained, 95% of which contained inserts, as judged by DNA miniprepations. The library was complete, as deduced from the number of clones obtained, and stable according to subsequent experiments with isolated clones. In contrast, *C. difficile* DNA has been reported to be unstable in some other vectors like λ gt11 (Muldrow *et al.*, 1987). All of the clones so far analysed carried inserts of less than 7.0 kb, with an average size of 5 kb.

The rabbit antiserum used for screening of the library specifically reacted with toxin A (Fig. 1) and was effective at dilutions of 1:72000 and 1:10000 in ELISA and immunoblot experiments, respectively. Fig. 1 also shows the cross-reaction of the mAbs 1339 and 1134 with toxin A and B (lanes 5–8). Screening of the library with the rabbit antiserum yielded 35 independent clones. *Bam*HI digestion of the plasmid DNA isolated from these clones showed that the sizes of the

Fig. 1. Reactivity of rabbit toxin A antibody and two mAbs against toxin A and B of *C. difficile*. In all the tests presented, 10 µg of toxin was separated on a 5–20% gradient SDS-PAGE gel and either stained with Coomassie blue (lane 1, toxin A; lane 2, toxin B) or transferred to nitrocellulose for immunoblotting (lanes 3 and 4, toxin A and B developed with the rabbit toxin A antiserum; lanes 6 and 5, toxins A and B developed with mAb 1339; lanes 8 and 7, toxins A and B developed with mAb 1134). The additional band at about 500 kDa in lane 2 (toxin B) is due to incomplete denaturation of the protein during incubation with the SDS-dye solution. It is converted into the 250 kDa band by denaturation for 10 min at 65 °C.

inserts varied between 2.5 and 7.0 kb. Neither culture supernatants nor total lysates of these clones exhibited cytotoxic activity in the CHO test. Mixtures of mAbs have been used successfully for identification of clones from a genetic library of mycobacterial DNA (Engers *et al.*, 1986). Screening of the *C. difficile* library with a mixture of four mAbs directed against toxin A did not permit the identification of positive clones. However, it is possible that these mAbs react with only one epitope (v. Eichel-Streiber *et al.*, 1987). Single mAbs not detecting their specific antigen within a genetic library have previously been reported (Young *et al.*, 1985). In the immunoblot analysis of total cell lysates of the recombinant clones, the toxin-A-specific mAb 1337 apparently detected the same protein bands as did the polyclonal rabbit toxin A antiserum (see Fig. 3 and data not shown).

Construction of a restriction map of overlapping clones

C. difficile DNA has a GC content of 28 mol % (Gottschalk *et al.*, 1981). In agreement with this finding, the cloned DNA was preferentially cleaved by restriction nucleases having AT-rich recognition sequences, e.g. *Bcl*I, *Dra*I, *Nde*I, *Ssp*I. All cloned segments were cut by *Rsa*I to produce several fragments. A restriction map was established for two representative overlapping clones pCd13 and pCd14 (Fig. 2). Enzymes cutting the inserts more than five times are not included in the map. The *Rsa*I sites in ten different overlapping clones are shown in Fig. 2. Hybridization of the pCd10 insert to *Rsa*I-cleaved plasmid DNA of the 35 positive clones demonstrated that they all contain overlapping DNA segments with a region of about 700 bp common to them all (shaded in Fig. 2). The occurrence of this common region is significant and possibly indicates that a major antigenic determinant is encoded within this region. The absence of antigenic structures in other parts of the holotoxin may explain why the complete toxin gene was not, apparently, isolated (see below).

Fig. 2. Physical map of recombinant clones hybridizing in the 10 kb region. The line immediately below the kb scale shows a partial restriction map of the 10 kb region: E, *EcoRI*; EV, *EcoRV*; H, *HindIII*; Hc, *HincII*; A, *AccI*; P, *PstI*; N, *NcoI*; Nd, *NdeI*. The following line shows *RsaI* fragments, numbered I–XIII according to their size. The remainder of the figure shows the inserts in the ten recombinant clones, aligned with the physical map; a 0.7 kb DNA segment common to all ten clones is shaded. All of the pCd clones shown carried the *EcoRI* and *SacI* sites of the pUC12 polylinker on the left side and the *BamHI* to *HindIII* sites on the right side of the aligned inserts. The vector pUC12 is omitted to simplify the figure.

Using pCd13 and pCd14 insert DNA as probes, Southern hybridization experiments were done with chromosomal DNA digested with a variety of restriction enzymes. The restriction fragments detected for the individual enzymes were in agreement with those outlined in Fig. 2 (with one exception: *PstI*, mentioned below), proving that the recombinant DNA originates from one part of the genome. There were seven *TaqI* sites within the 10 kb region characterized. Two *TaqI* fragments had a size of 0.3–0.4 kb; they were part of the *RsaI* fragments I and IV (Fig. 2 and data not shown). Muldrow *et al.* (1987) have cloned a 0.3 kb *TaqI* fragment in λ gt11; the expressed protein reacted with toxin A antibodies. This 0.3 kb fragment hybridized to a 4.5 kb *PstI* and a 16 kb *HindIII* fragment of *C. difficile* chromosomal DNA. Hybridization of pCd14 with *C. difficile* DNA digested with *HindIII* showed a band of similar size (see Fig. 5, lanes 3 and 4); hybridization to *PstI* fragments yielded two bands at 4.7 and 9 kb (data not shown). These results are in agreement with those presented by Muldrow *et al.* (1987). According to the restriction map in Fig. 2 (first line below the scale) there are two *PstI* sites with a spacing of 4.7 kb, but there is an additional *PstI* site between them, so that the 4.7 kb segment is cut into two fragments of 2.6 and 2.1 kb by *PstI*. Presumably, in *C. difficile* chromosomal DNA this *PstI* site is refractory to digestion, because of base methylation.

Expression of toxin-A-specific polypeptides by the recombinant clones

To characterize the proteins produced by the recombinant clones, total lysates were analysed by Western blotting (Fig. 3). Initially, only small amounts of protein could be detected with some clones. Therefore the sensitivity of the screening was increased using a sandwich technique including rabbit toxin A antiserum, goat anti-rabbit serum, and rabbit anti-goat-peroxidase

Fig. 3. Immunoblot analysis of recombinant clones expressing toxin A. Samples of toxin A (10 μ g) and of total lysates of the individual recombinant clones (50 μ g) were separated on a 5–20% SDS-PAGE gel and thereafter transferred to nitrocellulose. Proteins were detected using the polyclonal rabbit anti-serum specific for toxin A (see Fig. 1). Negative controls: lane 1, *E. coli* JM83; lane 2, *E. coli* JM83 transformed with pUC12. Positive control: lane 13, toxin A. Lanes 3–12, *E. coli* JM83 containing: 3, pCd14; 4, pCd27; 5, pCd21; 6, pCd22; 7, pCd17; 8, pCd15; 9, pCd16; 10, pCd33; 11, pCd10; 12, pCd13 (the protein extracts were submitted to PAGE analysis in the order of the orientation of the recombinant clones within the 10 kb region: cf. Fig. 2).

conjugate for the detection of proteins on the nitrocellulose. With this procedure proteins could be detected in all 10 clones analysed (Fig. 3). The proteins seen in the two control lanes (Fig. 3, lanes 1 and 2) are stained because of contaminating *E. coli* antibodies which are present in many antisera. The apparent size of the proteins (lanes 3–12) varied between 30 and 170 kDa as determined by PAGE and subsequent immunoblotting. Some of the multiple protein bands seen in Fig. 3 are probably due to premature termination of protein synthesis or protein degradation within the host strain. Similar results have also been obtained using a toxin-A-specific mAb (mAb 1337; data not shown).

Corresponding to a protein equivalent of about 140 kDa (cf. Fig. 3) the overlapping clones studied here apparently contained about 4 kb of toxin-A-specific DNA sequence. Based on the reported values of the molecular mass of the holotoxin, we therefore estimate to have cloned 1/4 to 1/2 of the toxin A gene. The isolated DNA segment expressing toxin A was smaller than expected from the reported molecular mass of the toxin; this was probably due to a lack of strong antigenic determinants in other parts of the toxin molecule. Alternatively, the small size of the DNA segment isolated might indicate that toxin A is composed of subunits, as has been proposed from a consideration of its reactivity towards one monoclonal antibody (Lyerly *et al.*, 1986a).

All recombinant clones expressing toxin A sequences contained the *C. difficile* DNA fragments inserted in the same orientation (Fig. 2). This suggests that transcription starts from an external promoter (*lac*) of the pUC vector. By cloning a 1.9 kb *Pst*I fragment from the toxin A gene carried by pCd14, into the *Pst*I site of the polylinker at the 3' end of the *cro'*-*lacZ'* gene on

Fig. 4. Western blot analysis of recombinant proteins derived from a *Pst*I fragment subcloned in pEX1, 2 and 3. A *Pst*I fragment from 2.6 kb to the right side of the common region was isolated from pCd14 and cloned into pEX1, 2 and 3 (see Fig. 2). Recombinant protein was isolated after induction (at 42 °C) of the recombinant *E. coli* strains harbouring the following plasmids: lanes 1–3, *Pst*I fragment in the correct orientation (lane 1, pEX1; lane 2, pEX2; lane 3, pEX3); lanes 4–6, *Pst*I fragment in the opposite (–) orientation (lane 4, pEX1; lane 5, pEX2; lane 6, pEX3). Lane A contains pure toxin A. Ten µg of toxin A and equal volumes (25 µl) of total lysates (25 µl) of the recombinant clones grown under identical conditions (see Methods) were separated on a 5–20% SDS-PAGE gel and thereafter transferred to nitrocellulose. The blot was stained with the toxin-A-specific mAb 1337.

pEX2, a hybrid gene was created, which encoded large amounts of a fusion protein of 170 kDa (Fig. 4). This is approximately the right size for a construction in which the *cro'*–*lacZ'* and the toxin-A-coding sequences are in frame. Trace amounts of the 170 kDa protein were also detected with the pEX1 and pEX3 derivatives, suggesting that during overexpression, translational frame shifting can occur as described by Stanley (1983). In addition, at least three minor proteins (29, 46 and 52 kDa) that reacted with the mAb1337 raised against toxin A were detected in pEX2. These proteins were also produced when the *Pst*I fragment was cloned into pEX1 or pEX3, in which the toxin A sequences are in different reading frames, indicating that the AT richness (72%) of the cloned *C. difficile* DNA induced reinitiation of translation, giving rise to these toxin A reactive proteins in pEX1, 2 and 3. When the *Pst*I fragment was inserted in the opposite orientation no recombinant proteins were produced, thus confirming the direction of transcription of the toxin A gene as being from left to right as shown in Fig. 2.

We calculated that the toxin-A-encoding DNA sequences as deduced from the largest protein detectable with the polyclonal antiserum in the Western blot (Fig. 3) extended from *Rsa*I fragment XII to I. According to this reasoning the coding sequence of all of the positive clones ends near the *Pst*I site of *Rsa*I fragment I, suggesting that the 3' end of the gene should be within this region. Deletion of sequences downstream of the *Pst*I site (at 4.8 kb) from pCd22, pCd17,

Fig. 5. Southern hybridization of radioactively labelled pCd14 insert DNA to digests of *C. difficile* chromosomal DNA at two different stringencies. Even-numbered lanes, $T_m - 15^\circ\text{C}$; odd-numbered lanes, reprobing of the identical nitrocellulose sheet at $T_m - 35^\circ\text{C}$. Lanes 1 and 2, *Rsa*I; lanes 3 and 4, *Hind*III; lanes 5 and 6, *Hinc*II; lanes 7 and 8, *Acc*I; lanes 9 and 10, *Eco*RI; lanes 11 and 12, *Eco*RV; lanes 13 and 14, *Sac*I. Minor differences in the labelled DNA fragments (lanes 7 and 8) are due to photographic reproduction.

pCd10 and pCd13 greatly reduced the abundance of the respective toxin-A-related proteins (data not shown), thereby suggesting that this region corresponds to a domain conferring stability and/or protease resistance upon toxin A.

Search for toxin-A-related genomic DNA

The clones pCd13 and pCd14, covering the entire 10 kb region, were used in hybridization experiments to screen for toxin-A-related sequences in the *C. difficile* genome. Insert DNA was hybridized to *C. difficile* chromosomal DNA digested with *Rsa*I, *Hind*III, *Hinc*II, *Acc*I, *Eco*RI, *Eco*RV, and *Sac*I, respectively. In these experiments variation of the stringency from $T_m - 13^\circ\text{C}$ to $T_m - 47^\circ\text{C}$ during hybridization did not lead to changes in the pattern of bands detected. A typical experiment, using pCd14 as a probe at 35°C and 15°C below T_m , is shown in Fig. 5. The 0.7 kb *Acc*I fragment shown in lane 7 was also seen in lane 8 on the original autoradiogram. The results indicate that additional sequences related to toxin A are not present in the *C. difficile* genome. The hybridization experiments indicate either that the toxins do not have any similarity, which would be surprising considering the cross-reactivity of the monoclonal antibodies, or that both toxins are encoded by a single gene. At present we cannot distinguish between these two possibilities. The search for a separate potential toxin B gene would require the preparation of a high-titre toxin B antiserum. At the present time such an antiserum is difficult to obtain due to the low immunogenicity and additional immunosuppressive effects of this potent toxin.

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