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Molecular Cloning and Nucleotide Sequence of the Alpha-Toxin (Phospholipase C) of *Clostridium perfringens*

RICHARD W. TITBALL,* SOPHIE E. C. HUNTER, KEITH L. MARTIN, BRIAN C. MORRIS, ANDREW D. SHUTTLEWORTH, TIM RUBIDGE, DEREK W. ANDERSON, AND DAVID C. KELLY

Chemical Defence Establishment, Porton Down, Salisbury, Wiltshire, SP4 0JQ, United Kingdom

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A fragment of DNA containing the gene coding for the phospholipase C (alpha-toxin) of *Clostridium perfringens* was cloned into *Escherichia coli*. The cloned DNA appeared to code only for the alpha-toxin and contained both the coding region and its associated gene promoter. The nucleotide sequence of the cloned DNA was determined, and an open reading frame was identified which encoded a protein with a molecular weight of 42,528. By comparison of the gene sequence with the N-terminal amino acid sequence of the protein, a 28-amino-acid signal sequence was identified. The gene promoter showed considerable homology with the *E. coli* σ^{55} consensus promoter sequences, and this may explain why the gene was expressed by *E. coli*. The cloned gene product appeared to be virtually identical to the native protein. A 77-amino-acid stretch that was close to the N terminus of the alpha-toxin showed considerable homology with similarly located regions of the *Bacillus cereus* phosphatidylcholine, preferring phospholipase C and weaker homology with the phospholipase C from *Pseudomonas aeruginosa*.

Clostridium perfringens is the etiological agent of a wide variety of diseases of animals. In humans there is a significant association of type A strains of the bacterium with wound infections, leading to gas gangrene. This is the disease caused by *C. perfringens* that is most studied in humans. The bacterium also causes a wide variety of other diseases such as necrotizing colitis in birds; necrotic enteritis in humans, calves, lambs, and pigs; and enterotoxemia in a variety of animal species (24). Strains of the bacterium can be classified as types A, B, C, or D, depending on the spectrum of toxins produced (24, 39); of these toxins the alpha-toxin has received the most attention. It was the first toxin for which an enzyme activity (phospholipase C) was implicated in conferring toxic properties on a bacterial product (20).

Although many different bacterial phospholipase C enzymes have been isolated, few are as toxic as the alpha-toxin, and this toxicity is thought to reflect two features of the protein; the enzyme exhibits a high turnover rate for phosphatidylcholine and sphingomyelin, which are key components of eucaryotic cell membranes (28). Also, unlike many other bacterial phospholipase C enzymes, alpha-toxin is readily able to interact with cell membrane phospholipids rather than monodispersed phospholipid suspensions (13, 30). These features combine to provide an index of phospholipase C toxicity: the ability of the enzyme to promote the hemolysis of erythrocytes (39).

Over the past 50 years, a wealth of data has accumulated concerning both the physiology of toxin production by the bacterium (39) and some of the biophysical properties of the protein (24). It has been suggested that the toxin plays a key role in the pathogenesis of *C. perfringens*-mediated gas gangrene infections when it promotes local cell membrane disruption (15, 39). In support of this, prior immunization against alpha-toxin can prevent the establishment of the disease (15). The alpha-toxin may also play a key role in the pathogenesis of other diseases such as necrotic enteritis of fowl, in which extensive damage to intestinal villi occurs. However, the difficulties associated with completely separating the alpha-toxin from the multitude of other potential toxins produced by the bacterium make these data difficult to interpret (29). These problems have also limited the use of the enzyme as a phospholipid-specific membrane probe (6, 41). We thought that the molecular cloning of the alpha-toxin gene into a suitable host bacterium would enable some of these problems to be resolved and, in addition, would enable us to examine the relationship of the protein structure to its function and antigenicity. Eventually, this will enable us to use immunogenic peptides or genes which encode toxoids to induce protection against alpha-toxin and some *C. perfringens* infections.

* Corresponding author.

MATERIALS AND METHODS

Enzymes and chemicals. Components for the preparation of growth media were obtained from Oxoid Ltd. (London, England) or Difco Laboratories (Detroit, Mich.). Restriction endonucleases, T4 DNA ligase, DNA polymerase I, Klenow fragment, and bacterial alkaline phosphatase were obtained from Boehringer Mannheim Biochemicals (Indianapolis, Ind.) and were used according to the instructions of the manufacturer. All other chemicals and biochemicals were obtained from Sigma Chemical Co. (St. Louis, Mo.) or BDH Ltd. (Poole, England), unless otherwise indicated. Chromatography materials for DNA or protein purification were purchased from Pharmacia Fine Chemicals (Piscataway, N.J.), unless otherwise stated.

Bacterial strains and cultivation. Clostridial strains were cultured anaerobically at 37°C in a liquid medium containing Trypticase Peptone (3%; BBL Microbiology Systems), yeast extract (0.5%; Difco), glucose (0.5%), cysteine hydrochloride (0.05%), and sodium thioglycolate (0.1%) that was adjusted to pH 7.5 by the addition of Na_2HPO_4 (TPYG broth). For the isolation of DNA or extracellular proteins, cells of *C. perfringens* NCTC 8237 were cultured in 6-liter volumes of TPYG medium for 18 h, and the cells and culture fluid were separated by filtration through a 0.22-μm-pore-size membrane (Millipore Corp., Bedford, Mass.).

Escherichia coli HB101, JM101, and CSH26 were stored

as glycerol stocks (22) and cultured on Luria agar (L-agar) or in L-broth (22) at 37°C. When necessary, the growth media were supplemented with ampicillin (50 μg/ml). *E. coli* Jm107 was maintained on a glucose-containing minimal medium (37). To induce and detect β-galactosidase production, isopropyl-β-D-galactopyranoside (320 μM) and 5-bromo-4-chloro-3-indolyl-β-D-galactoside (320 μg/ml) were incorporated into the solid growth medium.

Detection of alpha-toxin activity. Alpha-toxin activity was detected by determining its effect on egg yolk lipoproteins (increase in turbidity) or sheep erythrocytes (hemolysis). Egg yolk emulsion (Oxoid) was centrifuged before use (10,000 × *g*, 20 min, 4°C), and the supernatant was incorporated into the solid growth medium (10% [vol/vol]). Defibrinated sheep blood was centrifuged (1,000 × *g*, 5 min, 4°C), and the cell pellet was washed three times in phosphate-buffered saline (Oxoid) before it was finally suspended to its original volume in buffer. The washed sheep blood cells were then incorporated into the solid growth medium (5% [vol/vol]).

Alpha-toxin activity in liquid samples was detected by a microtiter tray assay. The samples (100 μl) were serially diluted across the microtiter tray in 100-μl volumes of dimethylglutaric acid-NaOH buffer (40), and after the addition of diluted egg yolk emulsion supernatant to each well (100 μl; 10% [vol/vol] in 0.9% saline), the microtiter tray was incubated at 37°C for 1 h. The phospholipase C activity of the original sample was expressed as the dilution which resulted in a 50% increase in the turbidity of the well contents and was expressed as egg yolk units per milliliter. For precise determinations, the optical densities at 620 nm of the microtiter tray well contents were determined with a plate reader (Flow Laboratories, Inc., McLean, Va.), and the 50% endpoint was calculated. Hemolytic activity was also determined by a microtiter tray assay. Freshly drawn citrated blood was washed three times by centrifugation in phosphate-buffered saline and was suspended in borate-buffered saline (0.2 M sodium borate , 7.5 g of NaCl per liter, 1.8 g of $CaCl_2$ per liter [pH adjusted to 7.6 with boric acid]) at 1% (vol/vol). Microtiter tray dilutions of samples were made as described above in borate-buffered saline, and 0.1-ml volumes of erythrocyte suspensions were added to each well. After incubation of the plates for 1 h at 37°C, the endpoint of this assay was considered to be the dilution causing 50% hemolysis of the erythrocytes (hemolytic units per milliliter). In some experiments the hemolytic titer was also read after incubation for an additional 1 h at 4°C.

Isolation and purification of DNA. DNA was isolated from the clostridia by the method of Marmur (23), but it was modified to include a final digestion stage with proteinase K (50 μg/ml) for 18 h at 37°C. DNA extracted from *C. perfringens* type A strain NCTC 8237 was additionally purified by centrifugation through a gradient of cesium chloride without ethidium bromide (22) and was finally dialyzed against three 1-liter volumes of 10 mM Tris hydrochloride buffer (pH 8.0) containing 1 mM EDTA (TE buffer) for 24 h at 4°C. The final preparation possessed an optical density at 260 nm/optical density at 280 nm ratio of 1.9.

Small-scale plasmid DNA isolations were performed by the method of Holmes and Quigley (11). For the isolation of larger quantities of chloramphenicol-amplified plasmid DNA (22), the method of Godson and Vapnek (8) was used; the DNA was then centrifuged through a cesium chloride density gradient (22).

Construction of a *C. perfringens* genomic library. The DNA that was isolated from *C. perfringens* type A strain NCTC 8237 was partially digested with the restriction endonuclease *Hin*dIII to obtain fragments mainly in the size range of 3.2 to 12.8 kilobase pairs (kbp) (mode, 5 kbp). The DNA fragments (1 μl) were mixed in a 1:1 molar ratio with an equal volume of *Hin*dIII-digested plasmid pUC18 (100 μg/ml), which was treated with bacterial alkaline phosphatase (50 U/μg, 30 min, 65°C), and 0.1 U of DNA ligase was added (2 μl). Following the addition of 1 μl of 4× ligation buffer (22), the ligation reaction was allowed to proceed for 18 h at 12°C. After transformation into competent *E. coli* JM101 cells (Stratagene), 3.1×10^5 ampicillin-resistant colonies were isolated per μg of plasmid.

Maxicell analysis of plasmid-encoded polypeptides. Recombinant plasmid pT5C100 was transformed into *E. coli* CSH26 cells that were made competent by the calcium chloride procedure (22). Plasmid-encoded proteins were radiolabeled with [^{35}S]methionine (1), and the polypeptides were examined after sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE). ^{14}C-labeled molecular weight marker proteins were obtained from Amersham Corp. (Arlington Heights, Ill.).

Nucleotide sequencing. The cloned DNA fragment encoding alpha-toxin was digested from its plasmid vector with *Hin*dIII and was isolated from agarose gels (0.7%) by electroelution. After suitable restriction endonuclease digestion, fragments were ligated with digested bacteriophage M13mp18 or M13mp19 and transfected into *E. coli* JM107 cells that were made competent by the calcium chloride technique (22). Recombinant phages were detected after the transfected cells were coplated with *E. coli* cells from a 2-h culture in L-broth onto L-agar plates containing isopropyl-β-D-galactopyranoside and 5-bromo-4-chloro-3-indolyl-β-D-galactoside. β-Galactosidase-negative plaques were picked, and after growth in *E. coli* JM107 for 6 h, the single-stranded M13 DNA was isolated by the method of Sanger et al. (37). Nucleotide sequencing was performed by the dideoxy chain termination method by using modified T7 DNA polymerase (Sequenase; USB Corp.), in accordance with the instructions of the manufacturer. Extension products were separated by electrophoresis in 6% polyacrylamide gels containing 46% (wt/vol) urea (Aldrich Chemical Co. Ltd.) and were visualized after exposure to X-ray film (Hyperfilm βmax; Amersham). Some sequencing reactions required the use of oligonucleotide primers other than the standard M13 primer (USB Corp.). These were prepared by using a DNA synthesizer (System 1+; Beckman Instruments, Inc., Fullerton, Calif.) and were then purified by ion-exchange chromatography (0.4 to 1.5 M NaCl gradient in 10 mM NaOH; Mono-Q fast-protein liquid chromatography [FPLC]), preparative polyacrylamide gel electrophoresis (20%), and centrifugation through columns of G15 Sephadex (22). The final DNA sequence was interpreted with a microcomputer system (DNAStar) and was compared with nucleotide sequences in the GenBank data base by using the NUSCAN program. The alpha-toxin amino acid sequence was compared with sequences in the DNAStar Protein Information Resource (PIR) data base by using the PROSCAN program. Hydropathy profiles of selected proteins were generated by using the PROTEIN program.

Fractionation of *E. coli* cells. *E. coli* HB101 cells containing plasmid pT5C100 were cultured for 20 h at 37°C in M9 medium (25) buffered with Tris hydrochloride (0.05 M, pH 7.4) and containing proteose peptone (0.2%), glucose (0.2%), $CaCl_2$ (100 mM), and L-proline (1 mM). The cells were harvested by centrifugation (10,000 × *g*, 20 min, 4°C) and were treated to extract the proteins that were located in the

periplasmic space or cytoplasm or membrane-bound proteins, as described by Minton et al. (26).

Purification of alpha-toxin. *C. perfringens* NCTC 8237 culture filtrate was concentrated from 6 liters to 500 ml by ultrafiltration through a membrane with a molecular weight cutoff of 10,000 (Millipore). The alpha-toxin was then precipitated with ammonium sulfate (50% saturation) for 1 h on ice, collected by centrifugation (10,000 × *g*, 20 min, 0°C), and redissolved in a minimal volume of 10 mM Tris–1% glycine buffer (pH 7.5; TG buffer). The material was applied to a gel filtration chromatography column (2.2 by 60 cm; Ultrogel ACA 44), and the column was eluted with TG buffer at a flow rate of 0.2 ml/min. Fractions (7 ml) containing alpha-toxin were pooled, incorporated into a flat-bed electrofocusing gel (pH 4 to 6.5; LKB Instruments, Inc., Rockville, Md.), and electrofocused at 18 W for 6 h at 10°C. Gel fractions were obtained by eluting proteins with TG buffer, and the alpha-toxin was finally separated from carrier ampholytes by elution from an FPLC gel filtration chromatography column (0.5 ml/min; Superose 12) with buffer (10 mM Tris, 5 mM $CaCl_2$, 2 mM $ZnSO_4$, 0.9% NaCl). The final preparations were stored at −20°C. In some experiments the alpha-toxin was purified by using two successive ion-exchange chromatography steps (flow rate, 1.5 ml/min; 20 mM triethanolamine hydrochloride buffer [pH 8]; 0.25 to 1.0 M NaCl gradient; FPLC Mono-Q column). The cloned alpha-toxin was extracted from the periplasmic space of cells cultured in 2-liter volumes of brain heart infusion broth and then purified by either the electrofocusing or the ion-exchange chromatography protocol described above.

Protein assays on purified toxin samples were performed by the method of Bradford (2), with bovine serum gamma globulin fraction used as the standard protein. The assay reagent was obtained from Bio-Rad Laboratories (Richmond, Calif.).

Amino acid sequencing. The N-terminal amino acid sequence of the purified *C. perfringens* alpha-toxin was determined with a gas-phase sequencer (470A; Applied Biosystems) equipped with an on-line phenylthiohydantoin (PTH) analyzer (120A; Applied Biosystems).

SDS-PAGE. Proteins were normally separated in polyacrylamide gels (7.5 or 10%) by using a discontinuous buffer system (18) and stained with Coomassie brilliant blue R250. When maxicell-encoded polypeptides were analyzed by SDS-PAGE, the gel was subsequently dried and exposed to X-ray film to detect radiolabeled plasmid gene products.

Molecular weight determination by gel filtration chromatography. Samples of purified native or cloned alpha-toxin were applied to an FPLC column (Superose 12) and eluted with 50 mM triethanolamine hydrochloride buffer (pH 8.0) containing 0.9% NaCl at a flow rate of 0.5 ml/min. The molecular weight of the alpha-toxin was determined by comparing its elution volume with those of proteins with known molecular weights (β-amylase, 200,000; bovine albumin, 66,000; carbonic anhydrase, 29,000; cytochrome *c*, 12,400).

RESULTS

Isolation of recombinant plasmids encoding *C. perfringens* alpha-toxin. *C. perfringens* type A strain NCTC 8237 (ATCC 13124) was the strain selected for cloning experiments because it has been the strain of choice for many previous studies on alpha-toxin. Chromosomal DNA from this strain was isolated and purified, and after digestion with *Hin*dIII it was ligated with suitably digested vector plasmid pUC18. After transformation of the resultant recombinant plasmids into *E. coli* JM101, approximately 2,000 ampicillin-resistant colonies were isolated, of which 86% were found to be β-galactosidase negative, suggesting that these clones contained plasmids with DNA inserts. When they were cultured onto L-agar containing ampicillin and egg yolk emulsion, two colonies containing recombinant plasmids pT5C100 and pT6C1534 were found to be surrounded by a zone of opacity, which is characteristic of the action of a lecithinase on this growth medium (21) (Fig. 1A). Colonies of both clones were also found to be surrounded by a zone of hemolysis when they were cultured on agar containing sheep erythrocytes. In contrast, *E. coli* JM101 containing pUC18 alone did not produce similar reactions. When clone T5C100 or T6C1534 was cultured onto solid medium which was previously spread with *C. perfringens* type A antitoxin, the zones of opacity (egg yolk agar) or hemolytic reaction (sheep erythrocyte agar) were not observed around the colonies (Fig. 1B). Normal serum did not cause a similar neutralization of these effects.

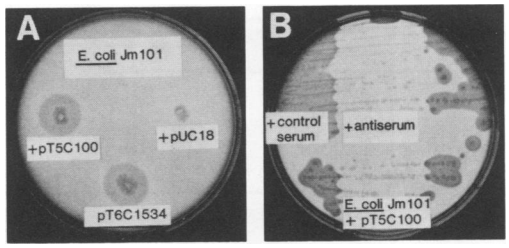

FIG. 1. Production of *C. perfringens* alpha-toxin (phospholipase C) by *E. coli* clones T5C100 and T6C1534. (A) *C. perfringens* type A genomic DNA was cloned into *E. coli* JM101 by using the plasmid vector pUC18. When the resultant clones were grown on agar containing egg yolk emulsion, two clones were isolated which were surrounded by a zone of turbidity (characteristic of phospholipase C activity). (B) When the alpha-toxin-producing clone T5C100 was cultured on agar containing egg yolk emulsion, which was previously swabbed with *C. perfringens* type A antiserum, complete inhibition of activity occurred.

Analysis of alpha-toxin-encoding recombinant plasmids. Phospholipase C (alpha toxin)-encoding recombinant plasmids (pT5C100 and pT6C1534) were isolated, digested with *Hin*dIII, and analyzed by agarose gel electrophoresis. Plasmid pT5C100 was found to make up pUC18 and a 3.1-kb insert, while pT6C1534 additionally contained inserts of 1.5, 1.2, and 1 kb. When the insert fragments from pT6C1534 were recloned separately into pUC18, only those recombinant plasmids containing the 3.1-kb DNA insert were found to encode the alpha-toxin. The size of the zone of opacity surrounding these colonies on egg yolk agar was judged to be similar to the size of the zones of opacity surrounding colonies of either T5C100 or T6C1534. The polypeptide-coding potential of the 3.1-kb DNA insert in plasmid pT5C100 was determined in a maxicell transcription-translation system. Plasmid pUC18 was found to encode a polypeptide with a molecular weight of 27,000 corresponding to the vector-encoded β-lactamase. Plasmid pT5C100 was found to encode a polypeptide with a molecular weight of 41,000 (mean of two determinations) and several polypeptides with molecular weights of 27,000 to 29,000, which may represent different forms of β-lactamase.

Physical mapping of the alpha-toxin-encoding DNA. The 3.1-kb *Hin*dIII DNA insert from recombinant plasmid pT5C100 was examined for the presence of restriction endonuclease digestion sites, and a physical map of the DNA

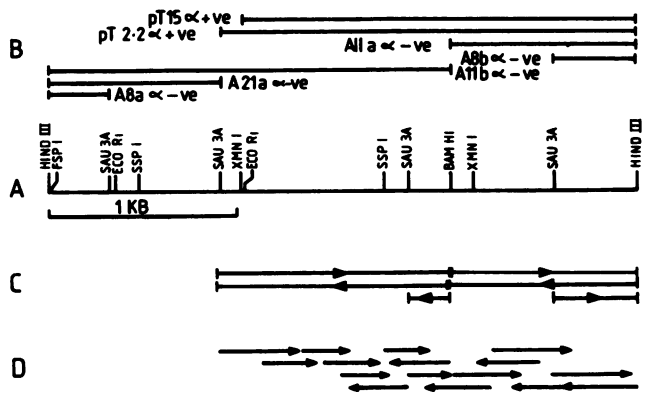

FIG. 2. Physical map of the 3.1-kb alpha-toxin-encoding DNA insert from plasmid pT5C100. (A) Restriction endonuclease digestion sites. (B) Alpha-toxin-positive and -negative subclones generated in plasmid pUC18. (C) Subclones generated in bacteriophage M13 for DNA sequencing. (D) Strategy used to determine the nucleotide sequence of the alpha-toxin gene.

fragment was constructed (Fig. 2A). The presence of a unique, asymmetrically placed *Bam*HI site enabled the orientation of the insert to be determined easily in recombinant plasmids generated by the ligation of the alpha-toxin-encoding insert with pUC18 vector plasmid. Expression of the alpha-toxin gene was shown to occur with the DNA insert in either orientation. In addition, when these clones were cultured on egg yolk agar, with or without isopropyl-β-D-galactopyranoside added (to induce the *lac* promoter), no change in the size of the zone of opacity surrounding the colonies was observed. These results indicate that the 3.1-kb DNA insert contains the alpha-toxin structural gene, together with its gene promoter, which is active within the *E. coli* host.

The physical map was also used to indicate restriction sites which could be used to generate subclones of the 3.1-kb alpha-toxin-encoding DNA; after partial digestion with *Sau*3A, the resultant fragments were ligated with suitably digested vector plasmid pUC18. A number of constructs were obtained (Fig. 2B); however, only recombinant plasmid pT2.2 (containing a 2.2-kb DNA insert) was found to encode the alpha-toxin. In later experiments, the coding region was further delineated by generating a 1.8-kb *Hin*dIII-*Eco*RI subclone of pT2.2 (Fig. 2B).

Nucleotide sequence determination of the alpha-toxin-encoding DNA. The nucleotide sequence of the 2.2-kb DNA insert which encoded the alpha-toxin was determined after suitable restriction fragments were subcloned into bacteriophage M13 (Fig. 2C). The sequencing strategy involved the use of synthetic oligonucleotide primers, when appropriate, to determine the nucleotide sequence of both strands within the gene promoter and protein-coding regions (Fig. 2D).

Examination of the nucleotide sequence (Fig. 3) revealed several open reading frames; however, all but one of these were much shorter than the predicted alpha-toxin-coding region. In addition, results of our maxicell experiments described above indicated that a single polypeptide was encoded by the DNA insert. An open reading frame extending from base 943 to base 2137 would encode a protein with a molecular weight of 45,480. The open reading frame was terminated by two consecutive TAA stop codons, with a third in-frame stop codon apparent 3 bases downstream.

Comparison of the putative coding sequence with the experimentally determined alpha-toxin N-terminal amino acid sequence revealed homology starting at nucleotide 1027. This may indicate the start of the mature alpha-toxin polypeptide. The amino acids encoded between the start codon and nucleotide 1027 could form a signal sequence directing transport of the alpha-toxin across the cell membrane. The proposed signal sequence would consist of a short charged N-terminal stretch followed by a hydrophobic core, which in turn would be followed by an amino acid stretch, fulfilling the criteria for a signal sequence cleavage site (47). Several other observations confirm this suggestion; the native alpha-toxin is known to be exported into the *C. perfringens* growth medium (40), and we found that the cloned protein is translocated across the *E. coli* cytoplasmic membrane into the periplasmic space.

If the alpha-toxin is synthesized as a precursor and is subsequently processed, then the calculated molecular weight of the mature toxin would be 42,528, which was in good agreement with our experimentally determined molecular weight of the native or cloned alpha toxins (43,300). Some differences were seen when the deduced amino acid composition of the protein was compared with the experimentally determined amino acid compositions reported previously. In particular, Mitsui et al. (27) failed to detect proline or cysteine within the alpha-toxin (Table 1), whereas we predicted that there are significant numbers of the former amino acid in the mature protein. The proportions of amino acids in the alpha-toxin, as determined by Krug and Kent (16), are comparable with our deduced composition. The acid hydrolysis step used by both groups of investigators (16, 27) would have promoted the destruction of tryptophan residues and prevented detection of this amino acid.

The coding region was found to contain 34% G+C residues, which is close to the reported *C. perfringens* G+C content of 24 to 27 mol% (3). As expected from this result, there was a marked preference for adenine or thymine in the first or third position of codon triplets.

The nucleotide sequence was examined for the presence of promoterlike sequences. Five consecutive G residues located 9 bases upstream of the proposed translation start point at position 943 could form a stable hybrid with the 3′ end of the *E. coli* 16S rRNA subunit (38), and such a duplex would be energetically favorable ($\Delta G = -10$ kcal) (43). This spacer sequence extending downstream to the translation start point showed many features that are normally associated with such a sequence: the region was rich in purine residues and contained no guanine residues at positions −1, −3, or −7 before the translation start point (48).

Upstream of the proposed Shine-Dalgarno region starting at base 863 there was a heptanucleotide stretch homologous with the *E. coli* σ^{55} RNA polymerase −10 consensus recognition sequence (TATAAT) (36). At 17 bases upstream from the proposed −10 recognition sequence, a pentanucleotide stretch showing 50% homology with the reported σ^{55} −35 consensus sequence (TTGACA) (36) was found. In addition, the nucleotide stretches adjacent to the proposed −10 and −35 recognition sequences showed considerable homology to the σ^{55} consensus sequence for these regions (36). Several other clostridial genes have been shown to possess *E. coli* σ^{55} promoterlike sequences (50); some, like the alpha-toxin −10 sequence that we have proposed, are completely homologous with the TATAAT consensus sequence, with much weaker homology with the −35 sequence. The 17-nucleotide spacing between the putative −10 and −35 recognition sequences of the alpha-toxin gene is similar to the optimal distance for strong gene expression in *E. coli* (36)

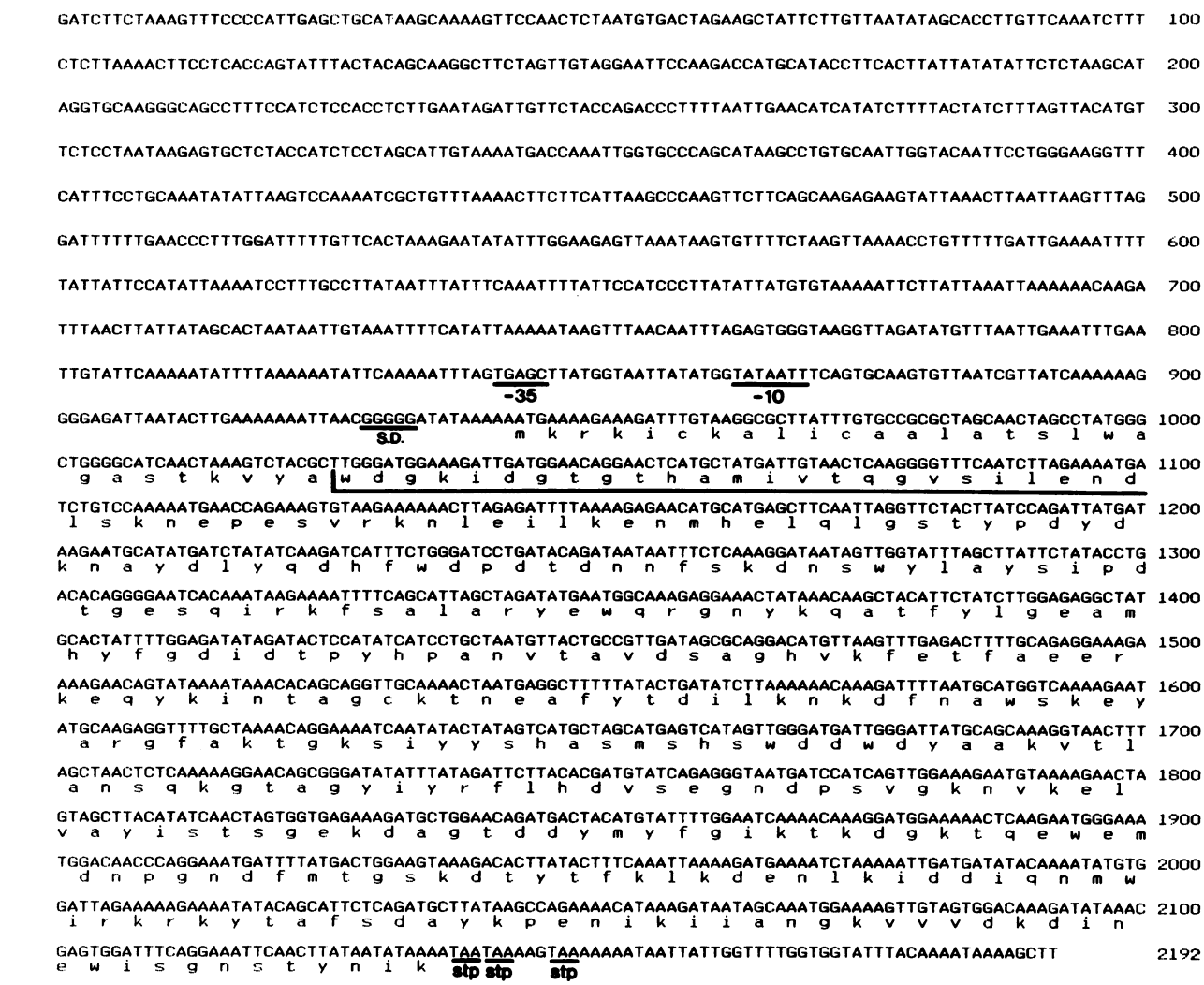

FIG. 3. Nucleotide sequence of the 2.2-kb alpha-toxin-encoding DNA fragment. The experimentally determined amino acid sequence of the N terminus of the mature alpha-toxin is boxed. Possible Shine-Dalgarno (S.D.) and –10 and –35 recognition sequences are underlined. stp, Stop codon.

and is similar to the reported spacer lengths in other clostridial genes (50).

A 31-base poly(AT) stretch observed upstream of the proposed –35 box was found to contain a tandemly repeated sequence TATTCAAAAAT and may play a role in the expression of the alpha-toxin gene. Similar AT-rich sequences have been observed upstream of genes that have been isolated from other gram-positive bacteria (31), and the function of such sequences may be to prevent interaction with histonelike proteins (32), which enhances the accessibility of the gene.

The nucleotide sequence downstream of the translation stop codon (position 2137) was examined for the presence of possible transcription termination signals. This stretch was found to have the potential to code for a number of short stem loops; a 6-base-pair stem loop centered at nucleotide pair 2173.5 may represent a [*rho*]-dependent termination signal; alternatively, a 4-base-pair stem loop centered at nucleotide pair 2164 and followed by a short poly(T) stretch may function as a [*rho*]-independent termination signal.

Alpha-toxin sequence homology with other phospholipases. No significant homology was detected between the mature alpha-toxin and the reported nucleotide or amino acid sequences of the *Staphylococcus hyicus* lipase (9) or any of the eucaryotic A2 or C phospholipases in the PIR (amino acid sequence) or GenBank (Los Alamos National Laboratory; nucleotide sequence) data bases. A significant level of amino acid homology has been found between the nontoxic phosphatidylcholine-preferring phospholipase C of *Bacillus cereus* (14) and the alpha-toxin; this homology appears to be maximal (34%) in an area containing histidine residues (D. Leslie, N. Fairweather, D. Pickard, G. Dougan, and M. Kehoe, submitted for publication). The phospholipase C of *Pseudomonas aeruginosa* showed little overall amino acid homology with the alpha-toxin, but a 77-amino-acid stretch was identified which was 26% homologous. The stretches of maximal homology among the *B. cereus* phospholipase C, the alpha-toxin, and the *P. aeruginosa* enzyme were all found close to the N terminus of the proteins and were located approximately in the same region (Fig. 4).

Further examination of structural homologies was undertaken by comparing the hydropathic potentials of the mature alpha-toxin and *B. cereus* enzymes predicted by using the methods of Hopp and Woods (12) or Kyte and Doolittle (17).

TABLE 1. Comparison of the predicted and experimentally determined amino acid composition of the mature *C. perfringens* alpha-toxin

Amino acid	No. predicted from DNA sequence[a]	No. experimentally determined by:	
		Mitsui et al. (26)[b]	Krug and Kent (16)[c]
Ala	26	30	30
Arg	9	10	9
Asn	26		29
Asp	35	55	36
Cys	1	0	2
Gln	10		11
Glu	23	40	26
Gly	25	35	29
His	9	15	8
Ile	22	20	22
Leu	16	20	17
Lys	36	40	39
Met	8	5	9
Phe	15	15	16
Pro	9	0	9
Ser	25	30	27
Thr	25	25	28
Trp	10	ND[d]	ND
Tyr	26	20	26
Val	14	15	15
Total	370	375	388

[a] Calculated from the nucleotide sequence of the cloned alpha-toxin gene.
[b] Data from Mitsui et al. (27), which were determined experimentally from purified *C. perfringens* alpha-toxin.
[c] Data from Krug and Kent (16), which were determined experimentally from purified *C. perfringens* alpha-toxin.
[d] ND, Not determinable.

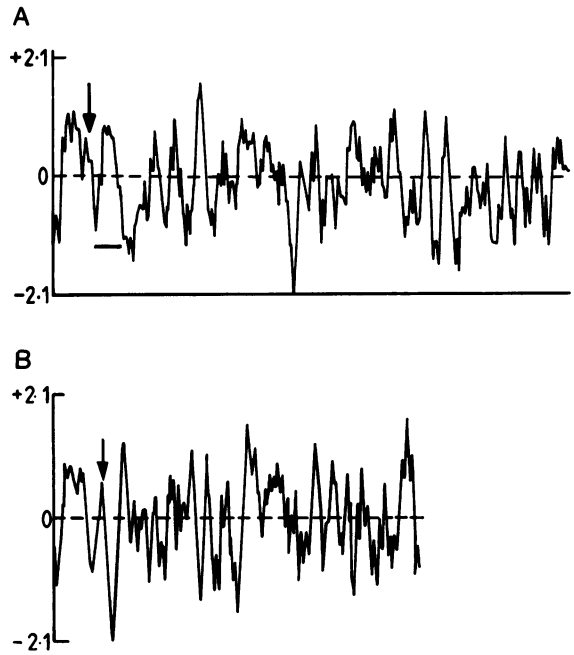

FIG. 5. Predicted hydropathic potentials of *C. perfringens* alpha-toxin and *B. cereus* phosphatidylcholine-preferring phospholipase C. The hydropathic potentials of alpha-toxin (A) and *B. cereus* phosphatidylcholine-preferring phospholipase C (B) were predicted by the method of Hopp and Woods (12). Hydrophobic (positive) and hydrophilic (negative) values are plotted on the *x* axis for each window of six residues. The probable signal sequence cleavage sites (alpha-toxin) or proenzyme activation cleavage sites (*B. cereus* enzyme) are indicated with arrows. The possible membrane-spanning N-terminal sequence in the mature alpha-toxin is underscored.

The alpha-toxin was predicted to possess a 13-amino-acid N-terminal hydrophobic region (Fig. 5A), while the nontoxic *B. cereus* enzyme possessed an N-terminal hydrophilic region (Fig. 5B).

The site of accumulation of cloned alpha-toxin in *E. coli*. The alpha-toxin-encoding recombinant plasmid pT5C100 was transformed into *E. coli* HB101, and a resultant clone was used to elucidate the site of accumulation of the cloned alpha-toxin in *E. coli*. The results (Table 2) indicated that most of the alpha-toxin produced was translocated across the cytoplasmic membrane and then accumulated within the periplasmic space of the bacterium. When proteins found in the periplasmic space were extracted and examined by SDS-PAGE, a major protein band was observed which migrated with the same mobility as the purified alpha-toxin (Fig. 6).

Purification of the native and cloned alpha-toxins and elucidation of their biophysical properties. The native *C. perfringens* alpha-toxin was purified by a combination of selective ultrafiltration, ammonium sulfate precipitation, gel filtration chromatography, and flat-bed electrofocusing. The cloned alpha-toxin was isolated from the periplasmic space of *E. coli* and then purified by the column chromatography and electrofocusing steps outlined above. The *C. perfringens* native alpha-toxin purified in this way was separated into a major form of the toxin with a pI of 5.48 and a minor form of the toxin with a pI of 5.6. In contrast, when the cloned alpha-toxin was fractionated by a similar technique, the form of the alpha-toxin with a pI of 5.6 was the major form that

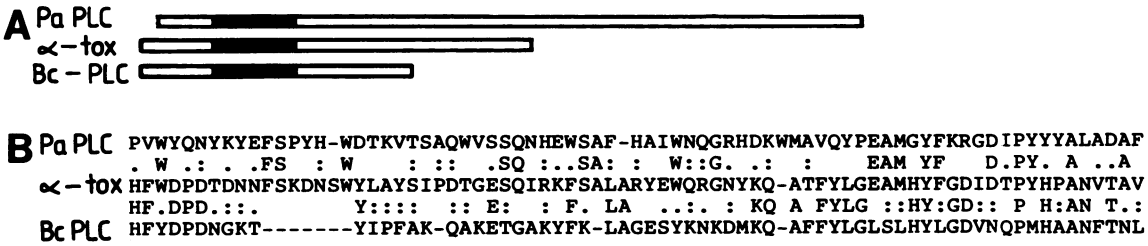

FIG. 4. Region of maximal homology between the phospholipase C enzymes of *C. perfringens* (alpha-toxin), *B. cereus* (phosphatidylcholine-preferring enzyme), and *P. aeruginosa*. (A) Location of regions of maximal homology (shaded) in the phospholipases C enzymes of *C. perfringens* (α-tox), *B. cereus*(BcPLC), and *P. aeruginosa* (PaPLC). (B) Alignment of amino acids within the region of maximal homology. PaPLC, *P. aeruginosa* phospholipase C, amino acids 52 to 129 (35); α-tox, *C. perfringens* alpha-toxin, amino acids 69 to 145; BcPLC, *B. cereus* phospholipase C, amino acids 69 to 146 (14). Double points indicate frequently substituted amino acids, and single points indicate rarely substituted amino acids.

TABLE 2. Site of accumulation of cloned alpha-toxin within cells of *E. coli* HB101[a]

Cellular fraction tested	Enzyme activity detected (% of total) in:			
	Alpha-toxin	Alkaline phosphatase	β-Galactosidase	NADH oxidase
Extracellular	1	0	0	0
Periplasmic	88	68	9	0
Cytoplasmic	11	29	78	5
Membrane-bound	1	3	13	95

[a] *E. coli* cells contained plasmid pT5C100 (pUC18 and a 3.1-kb DNA insert encoding *C. perfringens* alpha-toxin). Cells were grown for 20 h in a Tris-buffered liquid medium and were then treated under the conditions described in the text so that proteins would be released. The cell fractions were subsequently assayed for marker enzyme activities. The periplasmic space marker protein was alkaline phosphatase (44), the cytoplasmic marker protein was β-galactosidase (25), and the membrane-associated marker protein was NADH oxidase (33).

was detected (Fig. 7). The specific activities of the forms of the alpha-toxin with pIs of 5.48 and 5.6 were similar within an experiment, although variations between experiments were observed. The alpha-toxins with both forms of pI retained their identities when they were reexamined by using analytical electrofocusing, both in the presence and the absence of 6 M urea. In some experiments, minor protein bands with pI values of 5.15 and 5.3 were observed.

The molecular weights of both the native and cloned alpha-toxins were estimated to be 43,300 after the samples were examined by SDS-PAGE (Fig. 6) (mean of 13 determinations). No difference in the mobilities of the pI 5.48 or pI 5.6 forms of the native alpha-toxin was observed. When the molecular weight of similar alpha-toxin samples was determined by gel filtration chromatography, a much lower molecular weight was obtained (25,600; mean of three determinations).

The purified cloned alpha-toxin (100 μg/ml) was found to cause the hemolysis of murine erythrocytes (640 hemolytic units per ml) when tested at 37°C in a microtiter tray assay. The toxin showed no activity against horse, sheep, rat, or

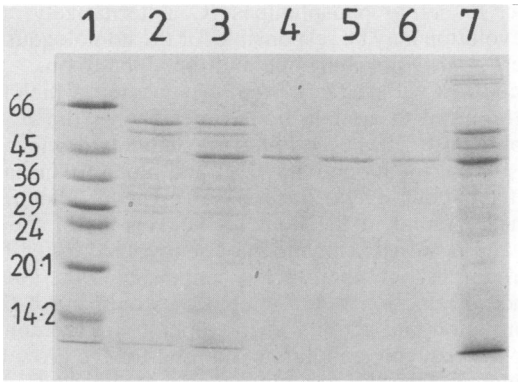

FIG. 6. SDS-PAGE (10%) of proteins extracted from the periplasmic space of *E. coli* and pUC18 (lane 2) or *E. coli* and pT2.2 (lane 3), purified forms of cloned alpha-toxin with pIs of 5.6 and 5.48 (675 ng; lane 4), the purified form of native alpha-toxin with a pI of 5.45 (610 ng; lane 5), the purified form of alpha-toxin with a pI of 5.6 (500 ng; lane 6), or *C. perfringens* extracellular proteins (lane 7). Lane 1 contains molecular weight markers, as follows: bovine albumin, 66,000; ovalbumin, 45,000; glyceraldehyde-3-phosphate dehydrogenase, 36,000; carbonic anhydrase, 29,000; trypsinogen, 24,000; trypsin inhibitor, 20,100; and α-lactalbumin, 14,200.

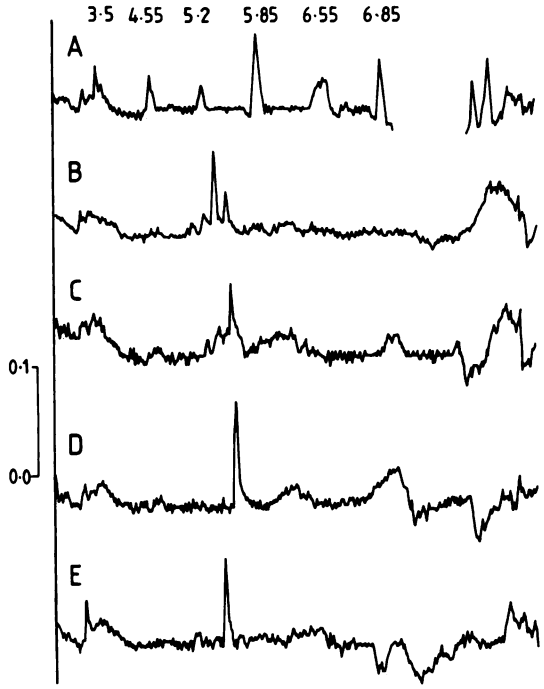

FIG. 7. Laser densitometer scan of analytical isoelectrofocusing gel (pH 3 to 9; Phast system; Pharmacia) stained with Coomassie brilliant blue R250. The bar on the *x* axis indicates an optical density at 633 nm of 0.1. (A) pI marker proteins; (B) purified native forms of alpha-toxin with mixed pIs; (C) purified cloned forms of alpha-toxin with mixed pIs; (D) purified native alpha-toxin with a pI of 5.6; (E) purified native alpha-toxin with a pI of 5.48.

rabbit erythrocytes. Some previous investigators (24) have found that the hemolysis of erythrocytes by the alpha-toxin is greatly increased if the erythrocytes are cooled to 4°C following the 37°C incubation step. We did not obtain a similar result with the purified cloned alpha-toxin; no increase in hemolytic titer was detected after any of the alpha-toxin-treated erythrocytes were cooled to 4°C for 1 h; in this respect our results are similar to those reported by Molby (28).

It has been reported that *C. perfringens* alpha-toxin is inactivated when it is heated to 60 to 70°C and is partially reactivated on further heating to 100°C (39). We obtained a similar result with the cloned alpha-toxin (36 μg/ml, 2,560 egg yolk units per ml); after the cloned alpha-toxin was heated to 60°C for 10 min in phosphate-buffered saline, only 12.5% of the initial phospholipase C activity was detected; however, after incubation for a similar period at 90°C, the enzyme retained 50% of the initial activity. A similar pattern of inactivation was observed when the hemolytic activity of the samples was determined against murine erythrocytes.

Initially, the immunological identity of the purified native or cloned toxin was assessed by an Ouchterlony double-diffusion technique. Alpha-toxin samples (forms with pIs of 5.48 or 5.6) were allowed to react with *C. perfringens* type A antiserum, and a single line of identity was observed between all of the samples tested (Fig. 8), indicating that some or all of the major epitopes are shared by the native and cloned toxins.

DISCUSSION

A segment of the *C. perfringens* genome which encodes the alpha-toxin gene was cloned into *E. coli* by using a

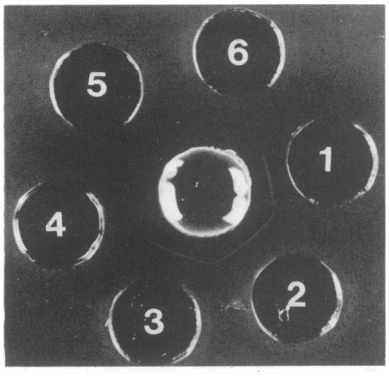

FIG. 8. Immunodiffusion reactions of *C. perfringens* type A antiserum (center well) against crude *C. perfringens* culture filtrate (well 1); purified *C. perfringens* alpha-toxin with a pI of 5.48 (well 2); or alpha-toxin with a pI of 5.6 (well 3), purified cloned alpha-toxin with a pI of 5.48 (well 4), alpha-toxin with a pI of 5.6 (well 5), or bovine serum albumin (well 6).

plasmid vector. Expression of the cloned gene appeared to be mediated by the associated promoter sequences. The pUC18 vector *lac* promoter appeared to play no role in the expression of the cloned gene. Examination of the nucleotide sequence of the cloned gene revealed potential σ^{55} −10 and −35 recognition sites (36) upstream of the protein-coding sequence and a 31-base poly(AT) stretch upstream of the proposed −35 sequence.

The translation termination point of the alpha-toxin gene was marked by repeated stop signals, a feature that is rarely observed within procaryotic genes and the significance of which is not known. The short stem loops identified downstream of the translation stop codon may act as transcription termination points; alternatively, the transcription terminator was located downstream of the fragment that we cloned and sequenced. Nucleotide sequence data for other clostridial genes suggests that [*rho*]-independent transcription stop signals are often used in this genus (4, 7).

Although the location of the proposed alpha-toxin promoter sequences would require experimental confirmation, we believe that the sequences we have suggested here and their relative positions would cooperatively result in gene expression. This was confirmed by the experimental results to some extent. Alpha-toxin is known to be one of the major extracellular products of *C. perfringens* (40), and we demonstrated expression of the alpha-toxin gene in *E. coli*. With the exception of *C. perfringens* bacteriocin (7), all other reported clostridial genes that have been cloned into *E. coli* have been expressed. The biased AT-rich codon usage was suggested as the reason for the lack of expression of the bacteriocin gene in *E. coli*, but we found that the codon usage in the bacteriocin and alpha-toxin genes is almost identical.

The alpha-toxin appeared to contain a typical signal sequence, and on export of the protein the active toxin was released into the culture supernatant. The phosphatidylcholine-preferring phospholipase C from *B. cereus* exhibits considerable amino acid sequence homology with the alpha-toxin (Leslie et al., submitted), and this protein possesses a signal sequence, although it is exported into the growth medium as a proenzyme requiring proteolytic cleavage of a 14-amino-acid N-terminal fragment for activation (14).

Our observation that *C. perfringens* alpha-toxin can exist in two major forms with different pIs is well documented (24), but we were unable to confirm the suggestion of Smyth and Arbuthnott (40) that 6 M urea can induce the conversion of these two forms to a single species. It is unlikely that the forms with different pIs are artifacts induced by the electrofocusing, because the two forms with different pIs retained their identities when they were individually refocused. The minor bands that we observed with pI values of 5.15 and 5.3 may be comparable to the minor forms of the toxin observed by Takahashi et al. (42). Microheterogeneity of bacterial toxins is a common observation and may result from a posttranslational modification, such as deamidation or phosphorylation of the protein. Our observation that the major pI form of the toxin is different when the gene is expressed in *E. coli* may indicate that such modification systems are not the same.

The calculated molecular weight of the mature alpha-toxin (42,528) is in close agreement with our experimentally determined value of 43,300. Previously reported molecular weight values determined by SDS-PAGE have been within the range of 41,000 to 90,000 (28), although more recent reports have indicated molecular weights closer to the former value (5, 16, 42, 49). Our results, indicating that the apparent molecular weight of the protein was much lower when determined by gel filtration chromatography, are also in agreement with previously reported results (24) and may be due to the hydrophobic interaction of the protein with the agarose-based support matrix.

We have predicted that there is only one cysteine residue per protein molecule, suggesting that intramolecular disulfide bonds cannot be involved in promoting structural stability, and it is of interest that the phospholipase C from *B. cereus* has also been reported to lack any disulfide bonds, yet it is also conformationally very stable (34). The observation by Krug and Kent (16) that alpha-toxin can exist in a minor form as an active dimer may reflect the formation of intermolecular disulfide bridges between the single cysteine residues in the protein.

Interpretation of the significance of the aligned homologous regions of the alpha-toxin with the *B. cereus* and the *P. aeruginosa* phospholipase C enzymes was made difficult by the observation that there was no direct homology between the *B. cereus* and *P. aeruginosa* enzymes. One explanation is that the proteins represent stages in the evolution of the smaller *B. cereus* phospholipase C. Alternatively, convergent evolution may be responsible for the homologous region of the *P. aeruginosa* enzyme and the alpha-toxin.

In contrast with the *P. aeruginosa* enzyme, which has not been reported to contain bound metal ions, the *B. cereus* phospholipase C has been found to contain two zinc ions that are essential for its activity (19), and alpha-toxin has been reported to contain two zinc ions (16). The histidine residues which are found in the homologous regions of the alpha-toxin and *B. cereus* enzyme may be involved in the binding of structurally or catalytically important zinc ions. The absence of zinc ion redox chemistry would mean that the conformation and activity of the alpha-toxin are conserved under the reducing conditions required for the growth of *C. perfringens*. The *P. aeruginosa* phospholipase C did not contain aligned histidine residues within the homologous region.

One difference between the alpha-toxin and the *B. cereus* phospholipase C is that although the *C. perfringens* enzyme is toxic, the *B. cereus* enzyme is not. This may reflect the reported ability of the alpha-toxin to damage a variety of eucaryotic cell membranes (28, 39). The purified cloned alpha-toxin that we tested represents the first preparation which is known to be completely devoid of other *C. perfrin-*

gens membrane-active proteins such as θ and δ toxins (24). The cloned alpha-toxin was capable of promoting the hemolysis of murine erythrocytes, which have previously been reported to be the most sensitive to hemolysis by alpha-toxin (24); but our observation that rabbit, sheep, and human erythrocytes are insensitive suggests that previous studies indicating that alpha-toxin can promote lysis of these erythrocytes when tested in a microtiter tray assay may, in fact, reflect the contamination of these preparations with other toxins. Our initial observation that weak hemolysis was detected around *E. coli* cells containing the alpha-toxin-encoding recombinant plasmid which were cultured on sheep erythrocyte agar may reflect the increased sensitivity of this detection method.

The ability of the alpha-toxin to interact with eucaryotic membranes may represent an important difference when compared with the interaction ability of the *B. cereus* phospholipase C. To examine this possibility, the hydropathic potential of the alpha-toxin was estimated; a potentially membrane-spanning hydrophobic stretch was found at the N terminus of the mature protein (Fig. 5A). Hydrophobic N-terminal sequences are thought to be important for the interaction of phospholipase A2 from pig pancreas (46) and human serum amyloid A protein (45) with lipid interfaces. The alpha-toxin N-terminal amino acid stretch could be involved in the binding of the alpha-toxin to cell membranes by becoming embedded in the lipid bilayer. The later mobility of membrane phospholipids would ensure that the bound enzyme was continually presented with substrate. When the hydropathic potential of the nontoxic phosphatidylcholine-preferring phospholipase C of *B. cereus* (14) was estimated, the N terminus of the mature protein did not possess a hydrophobic region (Fig. 5B).

These results provide a basis for further studies concerning the mode of action and immunogenicity of the alpha-toxin. We have generated monoclonal antibodies against the alpha-toxin, and they will be of use in determining the location of epitopes on the protein (manuscript in preparation). A study of the roles of specific amino acids in the activity of the protein is also in progress. The cloned gene product may be of use to other investigators as a phospholipid-specific membrane probe and for the evaluation of the role of this toxin in pathogenicity.

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