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Cloning and Nucleotide Sequencing of the *Clostridium perfringens* Epsilon-Toxin Gene and Its Expression in *Escherichia coli*

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The sequence of 20 amino acids from the N terminus of *Clostridium perfringens* epsilon-toxin was determined. Some differences between this sequence and the previously published sequence (A. S. Bhown and A. F. S. A. Habeeb, Biochem. Biophys. Res. Commun. 78:889–896, 1977) were found. A degenerate 23-bp pair oligonucleotide probe was designed from the amino acid sequence data and used to isolate a DNA fragment containing the gene encoding epsilon-toxin (*etx*) from *C. perfringens* type B. The gene encoded a protein with a molecular weight of 32,981. Upstream of the gene, promoter sequences which resembled the *Escherichia coli* σ^{70} consensus sequences were identified. The gene was expressed in *E. coli*, and the cloned gene product reacted with epsilon-toxin-specific monoclonal antibodies and had a molecular weight and isoelectric point similar to those of the native protein. Downstream of *etx*, two overlapping open reading frames were identified. Each encoded part of a protein which was homologous to the transposase from *Staphylococcus aureus* transposon Tn*4001*. Southern hybridization experiments indicated that the *etx* gene was found only in *C. perfringens* types B and D, the types which produce epsilon-toxin.

Clostridium perfringens is the causative agent of a wide variety of diseases. It has been associated with gas gangrene and food poisoning in humans and with a range of severe enterotoxemic diseases in many species of domestic animal. The bacterium produces up to 17 exotoxins (38) which are believed to play a role in pathogenesis, although in most cases their activity is poorly understood. The organism is grouped into five types (types A, B, C, D, and E) on the basis of the production of four major toxins, alpha-, beta-, epsilon-, and iota-toxins (6). Epsilon-toxin is produced by *C. perfringens* types B and D and is responsible for a rapidly fatal enterotoxemia in economically important livestock.

Epsilon-toxin has been purified by a number of independent workers, and its physical properties have been studied (reviewed in reference 22). It is secreted as a relatively inactive prototoxin which, after treatment with trypsin, is converted to fully active toxin with the loss of an N-terminal peptide (2). The mature toxin is a highly toxic protein with lethal, dermonecrotic, and edematic activities (1). However, it is not known how the toxin achieves its effects, although some amino acid residues important for its function have been identified. The chemical modification of amino acids has suggested that one tryptophan (30), one histidine (32), one tyrosine (33), three or four aspartic or glutamic acid (34), and eight lysine (31) residues are essential for the lethal activity of the toxin.

Despite the amount of information known about its physical properties and its biological activities, the mode of action of epsilon-toxin is not known, and no enzymatic activity has been attributed to it. In order to investigate its activity and to define the physical properties more precisely, we have cloned the gene (*etx*) encoding *C. perfringens* type B epsilon-toxin into *Escherichia coli*. This approach will also allow us to study the antigenicity of the protein and to examine the relationship between structure and function. Eventually, this could enable us to develop synthetic vaccines, based on immunogenic peptides or genetically engineered toxoids, which protect livestock against certain forms of enterotoxemia.

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MATERIALS AND METHODS

Enzymes and reagents. Materials for the preparation of growth media were obtained from Oxoid Ltd. or Difco Laboratories. All enzymes used in the manipulation of DNA were obtained from Boehringer Mannheim UK Ltd. and used according to the manufacturer's instructions. All other chemicals and biochemicals were obtained from Sigma Chemical Co. Ltd. unless otherwise indicated. Epsilon-prototoxin, purified from *C. perfringens* type D (strain unknown) was supplied by Wellcome Research Laboratories (Beckenham, Kent, United Kingdom). Anti-epsilon-toxin monoclonal antibodies, raised against a toxoid of the protein (5, 26), were supplied by D. Percival (Chemical and Biological Defence Establishment) or purchased from the Central Veterinary Laboratories (Weybridge, Surrey, United Kingdom).

Activation of epsilon-prototoxin. Epsilon-prototoxin was converted to active toxin with insoluble trypsin–L-1-tosylamide-2-phenylethyl chloromethyl ketone (trypsin-TPCK) attached to beaded agarose. Samples (100 μl) containing epsilon-prototoxin (up to 50 μg/ml) were incubated with 2.5 U of trypsin for 1 h at 37°C. The reaction was stopped by centrifugation to pellet the trypsin-TPCK.

Amino acid sequencing. Purified epsilon-prototoxin was converted to toxin with trypsin-TPCK. Samples of the toxin (100 pmol) were electroblotted on to a polyvinylidene difluoride membrane [Immobilon-P; Millipore (UK) Ltd.] from a denaturing polyacrylamide gel. The N-terminal amino acid sequence was determined directly from the blotted protein with a gas-phase sequencer (470A; Applied Biosystems) equipped with an on-line phenylthiohydantoin analyzer (120A; Applied Biosystems).

Bacterial strains and cultivation. *C. perfringens* strains were cultured anaerobically at 37°C in a liquid medium containing trypticase peptone (3%), yeast extract (0.5%), 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, cells of *C. perfringens* were cultured in 6-liter volumes of TPYG broth for 18 h and then separated from the culture fluid by filtration through a 0.22-μm-pore-size membrane. *E. coli* JM101 was stored and cultured as described by Sambrook et al. (35).

Preparation of DNA. Chromosomal DNA was isolated from strains of *C. perfringens*, *C. botulinum*, *C. novyi*, *C. bifermentans*, *C. histolyticum*, *C. sordellii*, *C. chauvoei*, *C. absonum*, *E. coli*, and *Bacillus subtilis* by the method of Marmur (21). DNA extracted from *C. perfringens* type B strain NCTC 8533 was purified by centrifugation through a gradient of cesium chloride (35). Large- and small-scale plasmid isolations were performed as described by Sambrook et al. (35). Fragments of DNA were isolated from agarose gels with DEAE cellulose paper (35). Oligonucleotides were prepared with a DNA synthesizer (200A; Beckman Instruments, Inc.). When used in hybridization, the oligonucleotides were synthesized without removing the final trityl group and then purified with an Oligonucleotide Purification Cartridge (Applied Biosystems) according to the manufacturer's instructions.

Blotting of DNA. After electrophoresis, DNA digested with restriction endonucleases was transferred to a nylon membrane (Zeta-Probe; Bio-Rad) with a vacuum blotting apparatus (Vacugene; Pharmacia LKB Biotechnology) according to the manufacturer's instructions. Undigested DNA was transferred to a nylon membrane with a slot blotting apparatus (Bio-Dot SF; Bio-Rad) according to the recommendations of the manufacturer.

Hybridization of gene probes. Oligonucleotide probes (10 pmol) were 5′ end labelled by using T4 polynucleotide kinase and [γ-^{32}P]ATP (35). The reaction buffer was modified to include 1 mM spermidine. Unincorporated [γ-^{32}P]ATP was removed by passing the labelled oligonucleotide through a Nensorb 20 column [DuPont (UK) Ltd.] according to the manufacturer's instructions. Isolated fragments of double-stranded linear DNA were labelled with [α-^{32}P]dCTP by using a random-primed labelling kit (Boehringer Mannheim UK). The labelled probes were hybridized to membranes as described by Sambrook et al. (35). For oligonucleotide probing, the temperature at which hybridization and washing took place was 42°C.

Cloning techniques. Chromosomal DNA from *C. perfringens* type B strain NCTC 8533 was digested with restriction endonucleases, ligated to 0.1 μg of digested and dephosphorylated pUC18 (Pharmacia LKB Biotechnology) (35), and transformed into *E. coli* JM101 cells (18). The cells were transferred to a nylon membrane (Hybond-N; Amersham International plc) by colony blotting as described by Sambrook et al. (35), except that after being replica plated, the colonies were incubated on L agar containing chloramphenicol (100 μg/ml) to amplify the plasmid DNA. After the membranes were probed with either radioactively labelled oligonucleotides or DNA fragments as described above, positive colonies were picked from the original plate and subcultured.

Nucleotide sequencing. Cloned DNA fragments which hybridized with *etx* gene probes were subcloned into bacteriophages M13mp18 and M13mp19. Templates for sequencing single-stranded M13 DNA (35) and plasmid DNA (9) were produced as described previously. Nucleotide sequencing was performed by the dideoxy-chain termination method with cloned T7 DNA polymerase (Sequenase version 2.0; United States Biochemical Corp.) according to the manufacturer's instructions. Nucleotide sequence and derived amino acid sequence data were analyzed with DNAstar microcomputer software. The amino acid sequences of epsilon-toxin and other open reading frames (ORFs) were compared with the Protein Information Resource data base of protein sequences by using the PROSCAN and AALIGN programs. Open reading frames were analyzed with the program FRAME (3), which runs on a MicroVAX 3600 computer.

Preparation of protein from cell cultures. *E. coli* cell lysates were prepared by sonicating cells resuspended in deionized water at 70% power with three 30-s pulses by using a Microson sonicator (Heat Systems Ultrasonics, Inc.). Alternatively, lysates were prepared by resuspending pelleted cells in deionized water and boiling them for 5 min in an equal volume of 2× gel loading buffer (35). *E. coli* HB101 cells containing plasmid pSH109 were grown for 16 h at 37°C in M9 medium buffered with 0.05 M Tris (pH 7.4) containing Proteose Peptone (0.2%), glucose (0.2%), and L-proline (1 mM). The cells were harvested by centrifugation (5,000 × *g*, 10 min, 4°C) and were treated to extract the proteins that were located in the periplasmic space or the cytoplasm as described by Minton et al. (24).

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Proteins were separated in 10 to 15% polyacrylamide gradient gels (Phastsystem; Pharmacia LKB Biotechnology) according to the manufacturer's instructions.

Isoelectric focusing. Proteins were separated in isoelectric focusing gels (pH 3-9; Phastsystem; Pharmacia LKB Biotechnology) according to the manufacturer's instructions.

Western blotting (immunoblotting). Proteins separated by SDS-PAGE and isoelectric focusing were capillary blotted onto a polyvinylidene difluoride membrane (Immobilon-P) according to recommendations of Pharmacia LKB Biotechnology. Membranes were blocked in 1% BLOTTO (35) and incubated with monoclonal antibodies specific for epsilon-toxin (5, 26) at a dilution of 1/1,000. Protein bands were visualized with an anti-mouse immunoglobulin G antibody, raised in goats and labelled with colloidal gold, at a dilution of 1/100 (Auroprobe BLplus; Cambio).

ELISA. Sandwich enzyme-linked immunosorbent assays (ELISAs) to detect epsilon-toxin contained in *E. coli* HB101 cell fractions were performed as previously described (26). The capture and detection monoclonal antibodies recognized different epitopes on epsilon-toxin. The detection monoclonal antibody was conjugated to horseradish peroxidase.

Nucleotide sequence accession number. The nucleotide sequence of epsilon-toxin gene from *C. perfringens* has been submitted to GenBank (accession number M80837).

RESULTS

Amino acid sequencing of epsilon toxin. Twenty residues were identified from the N terminus of *C. perfringens* type D epsilon-toxin, which could be partially aligned with the previously published N-terminal sequence of epsilon-prototoxin starting at Lys-14 (2) (Fig. 1). The homology between the two sequences after residue 20 was poor, although by removing Pro-23, residues 24 to 26 can be aligned.

Design of an oligonucleotide probe for the *etx* gene. Oligonucleotide probes designed from the previously published amino acid sequence of epsilon-prototoxin (2) failed to

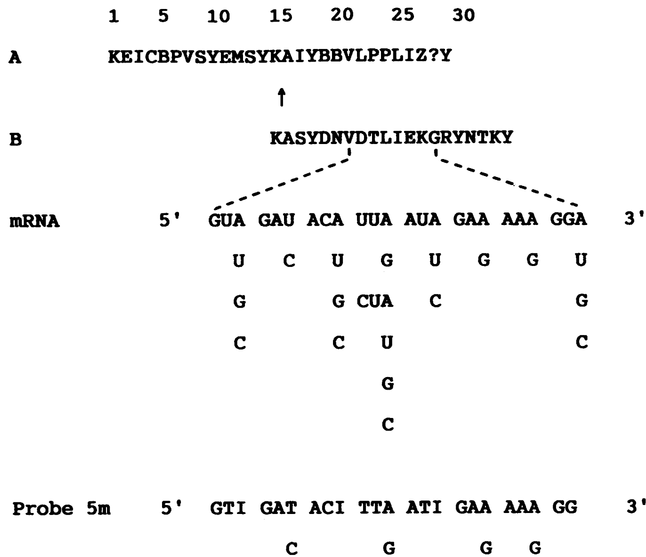

FIG. 1. The N-terminal amino acid sequence of *C. perfringens* type B epsilon-toxin was determined (B) and aligned with the sequence of *C. perfringens* type D epsilon-prototoxin determined by Bhown and Habeeb (2) (A). Residue B is Asp or Asn; residue Z is Glu or Gln. The site of trypsin cleavage reported by Bhown and Habeeb (2) is marked by an arrow. An oligonucleotide, 5m, was designed from the amino acid sequence (B) between residues 20 and 27; I is the base analog, deoxyinosine.

hybridize to DNA from *C. perfringens* types B and D. An oligonucleotide probe, 5m, was therefore designed from the new amino acid sequence data between residues 20 and 27 (Fig. 1). This sequence was of the same sense as mRNA and represented a region of minimal codon degeneracy. In order to reduce the redundancy of the oligonucleotide, the neutral base deoxyinosine was incorporated where more than two codons existed for an amino acid. In the case of Leu, the codons TTA and TTG were selected, since they were used on approximately 75% of occasions in *C. perfringens* genes. The resulting probe was 23 bp long and 16-fold degenerate and hybridized to the noncoding strand of the target sequence.

Hybridization of oligonucleotide 5m to *C. perfringens* DNA. Since the epsilon-toxin used in our sequence analysis was purified from an unspecified strain of *C. perfringens*, it was not possible to clone the gene from this source. Preliminary biochemical and immunological data (not shown) indicated that epsilon-prototoxins produced by *C. perfringens* types B and D were similar in terms of their physical properties, and we decided to clone the gene from a type B strain.

DNA from *C. perfringens* type B strain NCTC 8533 was digested with *Hin*dIII or *Sau*3A and probed with oligonucleotide 5m at 42°C. The hybridization temperature was calculated from the equation described by Suggs et al. (40) and allowed one mismatch between the probe and the target sequence to occur. Oligonucleotide 5m hybridized to 2.1-kb *Hin*dIII and 1.65-kb *Sau*3A restriction fragments.

Cloning of the epsilon-toxin gene. A library of *Hin*dIII-digested chromosomal DNA from *C. perfringens* NCTC 8533 was made in pUC18. After transformation, approximately 3,000 ampicillin-resistant colonies were obtained and were screened by colony hybridization using oligonucleotide 5m as a probe. Two positive colonies were identified, and recombinant plasmids pSH103 and pSH104 were isolated.

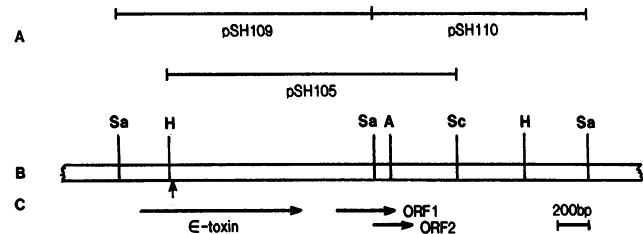

FIG. 2. Physical map of *C. perfringens* type B strain NCTC 8533 DNA in the region of the *etx* gene. (A) Clones generated in pUC18. (B) Restriction endonuclease digestion sites: A, *Acc*I; H, *Hin*dIII; Sa, *Sau*3A; Sc, *Sca*I. (C) ORFs identified. The vertical arrow indicates the site of hybridization of oligonucleotide 5m.

Restriction analysis of the plasmids revealed that pSH103 consisted of two vector molecules ligated to a 2.1-kb insert and that pSH104 contained two *Hin*dIII fragments with sizes of 2.1 and 2.2 kb. The fragments were recloned into pUC18. One of the recombinant plasmids (pSH105) contained a single 2.1-kb *Hin*dIII fragment which hybridized with probe 5m (Fig. 2A). DNA sequence analysis indicated that this fragment did not contain the entire coding region of the epsilon-toxin gene.

By restriction mapping and Southern blotting using oligonucleotide 5m as a probe, a physical map of the DNA in the region of the *etx* gene was obtained (Fig. 2B). The map indicated that the 1.65-kb *Sau*3A fragment recognized by probe 5m contained 330 bp upstream of the *Hin*dIII fragment cloned into pSH105. This *Sau*3A fragment was therefore large enough to encode the complete *etx* ORF.

With the *Hin*dIII insert from pSH105 used as a probe, *Sau*3A fragments in the region of the *etx* gene were isolated from a library of *Sau*3A-digested DNA from *C. perfringens* type B strain NCTC 8533 by colony hybridization. Four positive colonies were identified. Restriction analysis of the plasmids isolated from these clones showed that three of them (all pSH109) contained the 1.65-kb *Sau*3A fragment. The remaining plasmid (pSH110) contained a 1.25-kb *Sau*3A fragment located downstream of the *etx* gene (Fig. 2A).

Expression of the *etx* gene in *E. coli*. To compare the physical properties of the cloned and native epsilon-prototoxin, cell lysates of *E. coli* JM101 containing plasmid pSH109 or pUC18 were prepared by sonication. Samples of the lysates were treated with trypsin-TPCK to activate epsilon-prototoxin. The proteins were separated by SDS-PAGE or isoelectric focusing and analyzed by Western blotting with a monoclonal antibody, A5C12, which is specific for epsilon-prototoxin (26).

The molecular weights of cloned epsilon-prototoxin and epsilon-toxin were estimated to be 33,000 and 29,000, respectively (Fig. 3). Prototoxin and toxin purified from *C. perfringens* type D were found to migrate with similar mobilities. These results were confirmed by Western blotting with another anti-epsilon-toxin monoclonal antibody purchased from the Central Veterinary Laboratories (5) (data not shown).

The major pIs of cloned epsilon-prototoxin and epsilon-toxin were estimated to be 8.35 and 5.4, respectively, although a minor form with a pI of 5.0 was also present in the toxin (Fig. 4). Purified type D epsilon-prototoxin and epsilon-toxin also contained these isoelectric forms, although other forms were present as well.

Proteins with these properties were not found in the cell lysates prepared from *E. coli* containing pUC18.

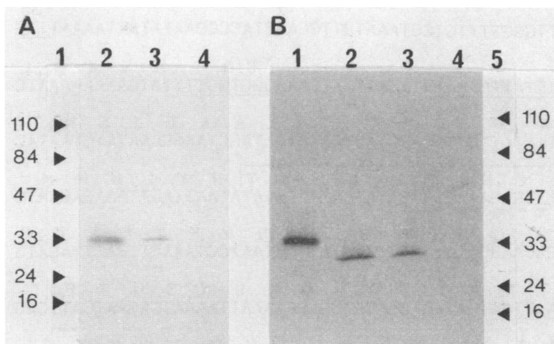

FIG. 3. Western blot of proteins separated by SDS-PAGE (10 to 15% polyacrylamide gradient gel [Phastsystem]) and probed with an anti-epsilon-toxin monoclonal antibody, A5C12 (26). (A) Lanes: 1, positions of protein molecular weight markers (10^3); 2, native epsilon-prototoxin; 3, *E. coli* and pSH109 cell lysate; 4, *E. coli* and pUC18 cell lysate. (B) Lanes: 1, native epsilon-prototoxin; 2, epsilon-toxin, prepared by treating native epsilon-prototoxin with trypsin; 3, trypsin-treated *E. coli* and pSH109 cell lysate; 4, trypsin-treated *E. coli* and pUC18 cell lysate; 5, positions of protein molecular weight markers (10^3).

Cell lysates of *E. coli* JM101 containing plasmid pSH109 were prepared by boiling at various times after inoculation. The lysates were analysed by SDS-PAGE and Western blotting with monoclonal antibody A5C12 (26). The concentration of epsilon-prototoxin in the culture reached a maximum after approximately 6 h of growth, corresponding to the late log phase (data not shown). The level of expression of epsilon-prototoxin was estimated to be 5 to 25 μg/ml of cell culture from quantitative Western blots using purified epsilon-prototoxin as concentration standards (data not shown).

Site of accumulation of cloned epsilon-toxin in *E. coli*. Plasmid pSH109 was transformed into *E. coli* HB101, and recombinant cells were fractionated to determine the site of accumulation of epsilon-toxin. The results (Table 1) indicated that most of the epsilon-toxin produced was translocated across the cytoplasmic membrane and was accumulated in the periplasmic space.

Nucleotide sequencing of the *etx* gene. Restriction fragments from recombinant plasmids pSH105, pSH109, and pSH110 were cloned into bacteriophage M13. M13 primers and synthetic oligonucleotide primers were used to determine the nucleotide sequence of the cloned DNA. An ORF extending from the ATG codon at base 188 to the TAA codon at base 1174 and containing a sequence identical to oligonucleotide 5m was identified (Fig. 5). The coding region had a high A+T content of 72.7%, which was consistent with the value of 69 to 73% previously reported for *C. perfringens* DNA (20). This bias was also reflected in the use of codons ending in A or T. For example, the codons GGA (Gly), UUA (Leu), AGA (Arg), and AUA (Ile) were used on 87.5, 73.7, 60, and 59.1% of occasions, respectively. In comparison, these codons are used on less than 10% of occasions in *E. coli* (25).

TABLE 1. Sites of accumulation of cloned epsilon-toxin in *E. coli* HB101 cells

Cell fraction tested	% of total activity detected		
	Epsilon-toxin[a]	Alkaline phosphatase[b]	β-Galactosidase[c]
Extracellular	0	2	1
Periplasmic	89	88	32
Cytoplasmic and membrane bound	11	10	67

[a] Assayed by sandwich ELISA using monoclonal antibodies specific for epsilon-toxin (26).
[b] Marker enzyme for the periplasmic fraction; the enzyme activity was assayed as described by Torriani (43).
[c] Marker enzyme for the cytoplasmic and membrane-bound fraction; the enzyme activity was assayed as described by Miller (23).

A purine-rich sequence, GGTGGT (bases 179 to 184), was located 4 bp upstream of the initiation codon (Fig. 5) and may be a ribosome binding site (36). Hybridization of this sequence to the 3′ end of 16S rRNA would be energetically favorable ($\Delta G = -10.2$ kcal [ca. -42.7 kJ] [42]). A hexanucleotide stretch, TATATT, was located between bases 120 and 125 (Fig. 5) and was 83% homologous to the -10 consensus sequence recognized by the major *E. coli* and *B. subtilis* σ factors (11). Sixteen base pairs upstream of the proposed -10 consensus sequence, there was another hexanucleotide stretch, TTGTAT, which was 50% homologous to the -35 consensus sequence. Between the proposed -10 consensus sequence and the Shine-Dalgarno sequence, there was also an AT-rich 10-bp direct repeat, TATAGAAAAA (Fig. 5).

The nucleotide sequence immediately downstream of the translation stop codon was examined for the presence of transcription termination signals (28). The absence of a significant stem-loop structure followed by a poly(T) stretch implied that rho-independent termination does not occur. However, 11 bp downstream of the stop codon, there was a short 11-bp stem-loop structure, incorporating one mismatch, which may be involved in the termination of transcription (Fig. 5).

Two short, overlapping ORFs downstream of the *etx* gene were also identified: ORF1 (bases 1360 to 1749) and ORF2 (base 1613 onward) (Fig. 2C and 5). The nucleotide sequence obtained did not include the 3′ end of ORF2. The ORFs were located on the same strand of DNA as the *etx* gene. Their nucleotide sequences were analysed with the program FRAME (3), which predicts ORFs on the basis of their nonrandom distribution of G and C bases in each codon position. This analysis suggested that the ORFs encode a

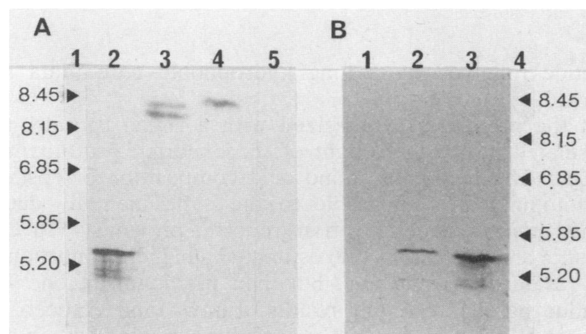

FIG. 4. Western blot of proteins separated in an isoelectric focusing gel (pH 3-9; Phastsystem) and probed with an anti-epsilon-toxin monoclonal antibody, A5C12 (26). (A) Lanes: 1, positions of pI marker proteins; 2, epsilon-toxin, prepared by treating native epsilon-prototoxin with trypsin; 3, native epsilon-prototoxin; 4, *E. coli* and pSH109 cell lysate; 5, *E. coli* and pUC18 cell lysate. (B) Lanes: 1, trypsin-treated *E. coli* and pUC18 cell lysate; 2, trypsin-treated *E. coli* and pSH109 cell lysate; 3, epsilon-toxin; 4, positions of pI protein markers.

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                                                                                                                    -35
 1    1 GATCGTTTTTAGTTCTATTTAAATAAACGATTTAATAATAAAAATTTTTAACTTGGGTTTTGTCGTAAATGTTGGAGCTACCCCAATATAATAAAATTTG

                             -10                                                                S.D.    M  K  K  N  L
 2  101 TATATTAAATAATTTTATTTATATTATTTACTTTTTTTAAAAAATATAGAAAAATATAGAAAAATATATTAATGAAAGGGTGGTTTTATGAAAAAAAATC

          V  K  S  L  A  I  A  S  A  V  I  S  I  Y  S  I  V  N  I  V  S  P  T  N  V  I  A  K  E  I  S  N  T
 3  201 TTGTAAAAAGTTTAGCAATCGCATCAGCGGTGATATCCATCTATTCAATAGTTAATATTGTTTCACCAACTAATGTAATAGCTAAGGAAATATCTAATAC

         V  S  N  E  M  S  K [K  A  S  Y  D  N  V  D  T  L  I  E  K  G  R  Y  N  T  K  Y] N  Y  L  K  R  M
 4  301 AGTATCTAATGAAATGTCCAAAAAAGCTTCTTATGATAATGTAGATACATTAATTGAGAAAGGAAGATATAATACAAAATATAATTACTTAAAGAGAATG

        E  K  Y  Y  P  N  A  M  A  Y  F  D  K  V  T  I  N  P  Q  G  N  D  F  Y  I  N  N  P  K  V  E  L  D  G
 5  401 GAAAAATATTATCCTAATGCTATGGCATATTTTGATAAGGTTACTATAAATCCACAAGGAAATGATTTTTATATTAATAATCCTAAAGTTGAATTAGATG

          E  P  S  M  N  Y  L  E  D  V  Y  V  G  K  A  L  L  T  N  D  T  Q  Q  E  Q  K  L  K  S  Q  S  F  T
 6  501 GAGAACCATCAATGAATTATCTTGAAGATGTTTATGTTGGAAAAGCTCTCTTAACTAATGATACTCAACAAGAACAAAAATTAAAATCACAATCATTCAC

         C  K  N  T  D  T  V  T  A  T  T  T  H  T  V  G  T  S  I  Q  A  T  A  K  F  T  V  P  F  N  E  T  G
 7  601 TTGTAAAAATACTGATACAGTAACTGCAACTACTACTCATACTGTGGGAACTTCGATACAAGCAACTGCTAAGTTTACTGTTCCTTTTAATGAAACAGGA

        V  S  L  T  T  S  Y  S  F  A  N  T  N  T  N  T  N  S  K  E  I  T  H  N  V  P  S  Q  D  I  L  V  P  A
 8  701 GTATCATTAACTACTAGTTATAGTTTTGCAAATACAAATACAAATACTAATTCAAAAGAAATTACTCATAATGTCCCTTCACAAGATATACTAGTACCAG

          N  T  T  V  E  V  I  A  Y  L  K  K  V  N  V  K  G  N  V  K  L  V  G  Q  V  S  G  S  E  W  G  E  I
 9  801 CTAATACTACTGTAGAAGTAATAGCATATTTAAAAAAAGTTAATGTTAAAGGAAATGTAAAGTTAGTAGGACAAGTAAGTGGAAGTGAATGGGGAGAGAT

         P  S  Y  L  A  F  P  R  D  G  Y  K  F  S  L  S  D  T  V  N  K  S  D  L  N  E  D  G  T  I  N  I  N
10  901 ACCTAGTTATTTAGCTTTTCCTAGGGATGGTTATAAATTTAGTTTATCAGATACAGTAAATAAGAGTGATTTAAATGAAGATGGTACTATTAATATTAAT

        G  K  G  N  Y  S  A  V  M  G  D  E  L  I  V  K  V  R  N  L  N  T  N  N  V  Q  E  Y  V  I  P  V  D  K
11 1001 GGAAAAGGAAATTATAGTGCAGTTATGGGAGATGAGTTAATAGTTAAGGTTAGAAATTTAAATACAAATAATGTACAAGAATATGTAATACCTGTAGATA

          K  E  K  S  N  D  S  N  I  V  K  Y  R  S  L  S  I  K  A  P  G  I  K  stp
12 1101 AAAAAGAAAAAAGTAATGATTCAAATATAGTAAAATATAGGAGTCTTTCTATTAAGGCACCAGGAATAAAATAAGATTATTTATTAGAAGTAAAAATAAG

13 1201 ATTTTAGTTTTATAGATTAATATTAATTCTAATAAAAACTCTATATAGATTTGTATGTAATCTAATTTTCCTCTTAAAGATAAATTAGACTTTCAAAATT

                                     -35                       -10      ¦ORF1 start              S.D.
14 1301 AAAACTTTGTAATCTTAAGTCTAATTTGAGAGTGTAAAATAGTCTGTGTAAACTAATAAGATGATATAATTAAATATCATATAAAAGGAGGGTGCACAGA

15 1401 CTATATCTTATGCCGAAAAAGAATTGATAAAACAACTAATATAGAGACTGCTGAATATGCTCAAAATGTTATTAAAAGTTTATTTGGTGGTTTAATTCAA

16 1501 CAAATACTTGAAGCTGAAATGGAAGAATATTTAGGATATTTAAAATATGATTATTCAAATAAAAATACTACTGATTCTCGTAATTGGGAAAATGAGAAAA

                   ¦ORF2 start
17 1601 CTGTTAAATTTGATTTAGATATCCCAAGAGATCGTGAGGGTTCTTTCGAACCACAAATCGAAAAGAAACATCAAACTACTAGAAAGTTCAATAATAGGGG

                                                       ORF1 end ¦
18 1701 GGAGGTATGCGAAAGGCATGTCTACACGAGATATAGCTACTCAAGTTAATGAAATGTACGGCATGGATGTTTCACCTACTTTAATCTCAAATATAACTGA

19 1801 TAAAATAATACCTTCTATAAAAGAATGGCAATGTAAACCATTAGAAAGTATATATCCTATCG
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FIG. 5. Nucleotide sequence of the DNA fragment encoding epsilon-toxin. The data from bases 1 to 1633 were obtained from both strands of the cloned DNA in pSH105 and pSH109; the remaining data were obtained only from the noncoding strand of the pSH110 insert, but three M13 subclones were sequenced independently. The nucleotide and derived amino acid sequences of the *etx* gene between the start codon at base 188 and the stop codon (stp) at base 1174 are shown. The experimentally determined N-terminal amino acid sequence of epsilon-toxin that we obtained is bracketed. The N-terminal peptide of epsilon-prototoxin that is cleaved by trypsin is shown in boldface type. The nucleotide sequence of oligonucleotide 5m is indicated with a dashed line. The overlapping ORFs, ORF1 and ORF2, situated downstream of the *etx* gene are shown. Possible Shine-Dalgarno (S.D.) and −10 and −35 consensus sequences controlling the transcription and translation of the *etx* gene and ORF1 are indicated. Direct repeats and inverted repeats associated with the *etx* gene are underlined with half-arrows.

single gene disrupted by a frameshift mutation in the region of bp 1680 to 1710 (data not shown). However, none of the possible start codons, including AUG, GUG, AUA, ACG (37), and UUG (27), was present at the 5′ end of ORF1, although sequences resembling a Shine-Dalgarno region and the *E. coli* σ^{70} −10 and −35 consensus sequences were identified (Fig. 5).

Analysis of the derived amino acid sequence. The amino acid sequence derived from the *etx* gene was identical to the N-terminal amino acid sequence of epsilon-toxin (as reported here) over 20 residues, starting from Lys (base 323) (Fig. 5). Of the 13 amino acids preceding this residue, 9 were aligned with the previously published N-terminal protein sequence of epsilon-prototoxin (2), suggesting that Lys (base 284) may be the first residue of the mature, inactivated protein. The 32 amino acids from the start Met to this Lys contained residues characteristic of a signal peptide (44), and plots of the hydropathic potential of the protein indicated that this region consisted of a short, charged N-terminal peptide followed by a long hydrophobic core (data not shown).

If the protein is synthesized with a signal peptide, the calculated molecular weight of the exported prototoxin is 32,981. The predicted amino acid composition of epsilon-prototoxin was comparable to the experimentally determined amino acid compositions reported previously (16, 29). Sakurai and Nagahama (29) estimated a higher proportion of the residues Ala and Gly, but their prediction of one Trp residue agreed with our results. Bhown and Habeeb (2) reported that the single Cys residue was located in the N-terminal peptide which is lost after trypsin cleavage, whereas we predicted that it occurred in the central region of the protein (Fig. 5).

Computer analysis of the secondary structure of epsilon-prototoxin predicted that the protein consisted of 52% β-sheet according to the algorithm of Garnier et al. (14) and up to 70% β-sheet according to the Chou and Fasman algorithm (10). This confirmed previous results derived from

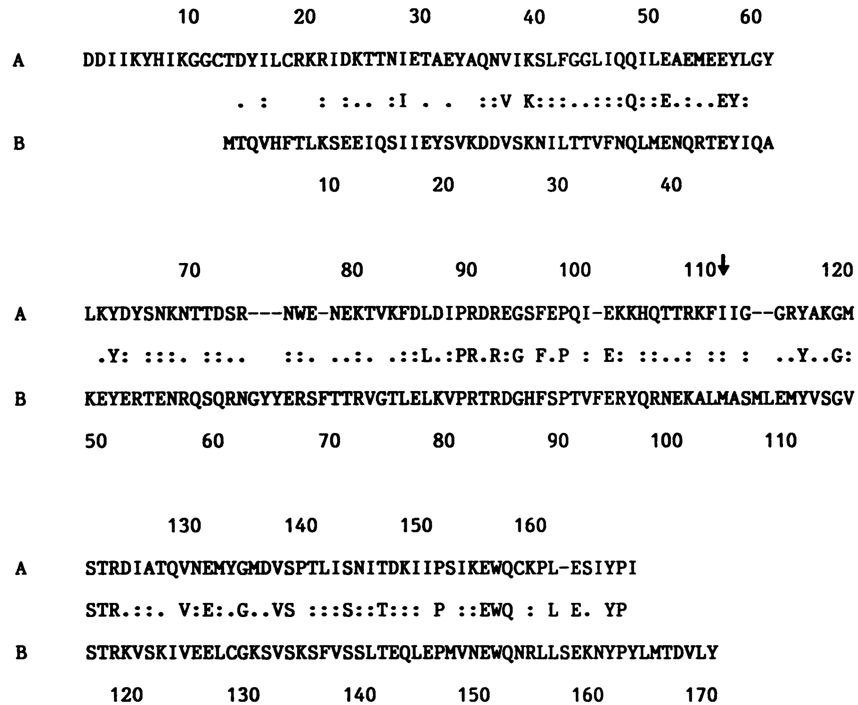

FIG. 6. Alignment of the amino acids of the ORF1-ORF2 fusion protein (A) and amino acids 1 to 163 of the transposase from *S. aureus* transposon Tn*4001* (8) (B). The arrow indicates the point of fusion between amino acids derived from ORF1 and ORF2. Double dots indicate frequently substituted amino acids, and single dots indicate rarely substituted amino acids.

circular dichroism and optical rotatory dispersion studies, which also indicated that the protein consisted predominantly of β-sheet (17). A computer search of the Protein Information Resource data base did not reveal significant homology (>20%) with any other proteins, so we were unable to make further detailed predictions about the structure or function of epsilon-toxin.

The nucleotide sequences of ORF1 and ORF2 were fused at position 1690 (Fig. 5), on the basis of the assumption that a frameshift mutation occurred in the region consisting of bp 1680 to 1710. The derived amino acid sequence was also compared with those in the Protein Information Resource data base. It was found to be 24% homologous to the transposase from *Staphylococcus aureus* transposon Tn*4001* (8), although the C-terminal region of the ORF1-ORF2 fusion (position 86 to 166) exhibited a higher degree of homology (33%) (Fig. 6).

Probing clostridial DNA with the *etx* gene. Recombinant plasmid pSH109 was digested with restriction enzymes to yield either a 1.65-kb *Sau*3A fragment or a 1.3-kb *Hin*dIII-*Sau*3A fragment containing the *etx* gene (Fig. 2).

In slot blotting experiments, the *etx* gene probe hybridized strongly to DNA from *C. perfringens* types B and D only (data not shown). Weak hybridization with DNA from *E. coli* JM101 was also seen; this may be due to contamination of probe DNA with *E. coli* chromosomal DNA. The gene probe did not hybridize to DNA from *C. perfringens* types A, C, and E, *C. botulinum*, *C. novyi*, *C. bifermentans*, *C. histolyticum*, *C. sordellii*, *C. chauvoei*, *C. absonum*, or *B. subtilis*.

In Southern blotting experiments with DNA from *C. perfringens* types B and D, the *etx* gene probe hybridized to 1.65- or 3.25-kb *Sau*3A fragments (Fig. 7A) and 4.5- or 5.6-kb *Xba*I fragments (Fig. 7B). These results indicated that the restriction profiles of *C. perfringens* types B and D varied in the region of the *etx* gene, although the fragment sizes recognized were not type specific and differed only between strains.

DISCUSSION

The epsilon-toxin gene from *C. perfringens* type B has been cloned and expressed in *E. coli* by using plasmid vector pUC18. Expression of the protein was probably controlled by an *etx* promoter, since the gene was not cloned in the correct orientation to be expressed from the pUC18 *lac* promoter. Signals potentially controlling transcription and translation were identified in the cloned fragment, but because of the high A+T content of the DNA, caution must be exercised in identifying promoter elements, particularly those recognized by *E. coli* σ^{70} and *B. subtilis* σ^{43}, merely by sequence inspection. It is possible that the −10 and −35 sequences that we identified were used fortuitously in *E. coli* and that other sequences are used in *C. perfringens*. The function of the 10-bp direct repeat located upstream of the proposed ribosome binding site is unknown, although it may also be involved in controlling gene expression.

The gene was expressed at relatively low levels, perhaps because of instability of the product or inefficient transcription and translation. Although product instability has reduced the expression of some clostridial toxins (13), we did not detect any degradation products of cloned epsilon-toxin. Biased codon usage in clostridial genes has been reported to hinder the efficiency of translation in *E. coli* (15), although this effect would be minimal in a relatively small gene such as *etx*.

The expressed protein accumulated in the periplasmic space of *E. coli*, suggesting that it was produced with a signal

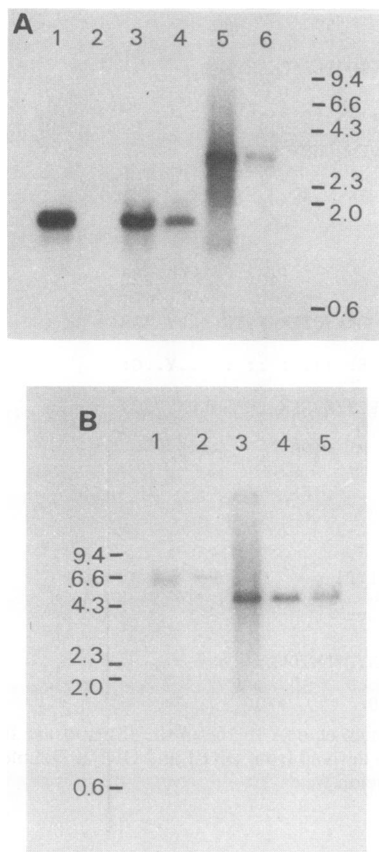

FIG. 7. Southern blot of *C. perfringens* type B and D DNA (1 to 3 μg) digested with *Sau*3A (A) or *Xba*I (B). The DNA was probed with an [α-^{32}P]dCTP-labelled *etx* gene probe. (A) Lanes: 1, 0.03 μg of plasmid pSH109; 2, blank; 3, type B strain NCTC 8533 DNA; 4, type B strain NCTC 6121 DNA; 5, type B strain CN 8024 DNA; 6, type D strain NCTC 8346 DNA. (B) Lanes: 1, type B strain NCTC 8533 DNA; 2, type B strain NCTC 6121 DNA; 3, type B strain CN 8024 DNA; 4, type D strain NCTC 8503 DNA; 5, type D strain NCTC 8346 DNA. The positions of DNA molecular size markers are indicated in kilobases.

peptide which allowed it to cross the cytoplasmic membrane. This was supported by the fact that *etx* encoded a sequence which was structurally similar to a signal peptide; also, it is known that the native protein is exported from *C. perfringens* (7).

We have shown that the cloned type B and native type D toxins have similar molecular weights and isoelectric points and that they both react with specific monoclonal antibodies. The similarity of their properties is supported by the fact that the amino acid sequence derived from the cloned type B gene is identical to the amino acid sequence of the type D toxin that we obtained over 20 residues. The amino acid differences in the previously reported sequence of type D epsilon-toxin (2) may therefore be due to inaccuracies in sequence analysis. This may be partly explained by the fact that epsilon-toxin has proved difficult to purify (19) and may contain contaminating proteins and breakdown products which interfere with sequence analysis.

The molecular weight of mature epsilon-prototoxin calculated from the derived amino acid sequence (32,981) agreed with our experimentally determined value for the cloned and native proteins of 33,000. Previously, a range of molecular weights for the protein have been reported (from 23,200 to 40,500 [22]), although more recent estimates (29, 45) are much closer to our value.

The isoelectric points of epsilon-prototoxin and epsilon-toxin have been reported to be 8.02 and 5.36, respectively (45). The estimated pIs of cloned prototoxin (8.35) and toxin (5.4) differ slightly from those of the previous reports but are identical to the major pIs of the purified proteins from *C. perfringens* type D, which we have determined. Our method of estimating the pI is not precise and may account for the observed differences. It relies on capillary transfer of the proteins to a membrane after isoelectric focusing, and during this time, the proteins can migrate away from their isoelectric points. Other isoelectric forms of the type D prototoxin, with intermediate pIs, were observed. Previously, it has been shown that these forms are more toxic than the prototoxin but less toxic than the toxin (45), suggesting that activation is a multistep process involving sequential removal of short peptides from the N terminus of the prototoxin. Minor isoelectric forms of epsilon-toxin, with pIs of <5.4, were also seen. They may also represent degraded forms of the protein, or they may result from posttranslational modifications, such as deamidation or phosphorylation. Although multiple isoelectric forms of epsilon-toxin (rather than epsilon-prototoxin) have not been reported previously, microheterogeneity of bacterial toxins has been frequently observed (41).

Although we were unable to make detailed predictions about the structure and function of epsilon-toxin, hydrophobicity plots of the protein (not shown) indicated that there were no significant hydrophobic stretches, apart from the proposed signal peptide. It is therefore unlikely that epsilon-toxin is a membrane-spanning protein.

Bhown and Habeeb (2) reported that the trypsin cleavage site in epsilon-prototoxin lay between Lys-14 and Ala-15 (Fig. 1). However, the amino acid sequence data derived from the *etx* gene indicated that the residue preceding Lys-14 was also Lys. Since trypsin cleaves proteins on the C-terminal side of basic amino acids, this suggested that there were two possible sites for cleavage and may explain why we found that the N-terminal residue of epsilon-toxin was Lys-14, not Ala-15 as previously described (2).

Chemical modification studies have suggested that one Trp (30), one His (32), one Tyr (33), three or four Asp or Glu (34), and eight Lys (31) residues are essential for the lethal activity of epsilon-toxin. From the derived amino acid sequence, it was not possible to identify which of the 17 Tyr, 33 Asp or Glu, and 29 Lys residues in the prototoxin may be functionally important. In contrast, only one Trp residue and two His residues were found in the derived amino acid sequence, so these amino acids are a natural target for studying structure-function relationships of epsilon-toxin.

The variability of the restriction profiles of *C. perfringens* types B and D in the region of the *etx* gene may be explained by the presence of ORF1-ORF2, which may encode part of a transposon, downstream of the gene. Although Southern blotting experiments indicate that ORF1-ORF2 is found in all of the *C. perfringens* strains tested (data not shown), regions of DNA containing transposons tend to be variable and may contain other mobile elements.

We have shown that the *etx* gene is found only in *C. perfringens* types B and D, the types which produce epsilon-toxin. This may suggest that the gene is carried on an extrachromosomal element not found in the other types. Preliminary results from mapping the genome of *C. perfringens* types B and D have indicated that the gene is carried on

a large plasmid (>100 kb) (8a). It is known that *C. perfringens* contains a large number of plasmids (39), and there is evidence that other *C. perfringens* toxins, including type B beta-toxin (12) and type B lambda-toxin (4), are plasmid borne. Since *etx* is present only in the types producing epsilon-toxin, the cloned gene will be an important tool for classifying *C. perfringens* strains and diagnosing some forms of enterotoxemia.

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