Molecular Genetic Analysis of Beta-Toxin of *Clostridium perfringens* Reveals Sequence Homology with Alpha-Toxin, Gamma-Toxin, and Leukocidin of *Staphylococcus aureus*

SOPHIE E. C. HUNTER,[1] J. EDWARD BROWN,[2] PETRA C. F. OYSTON,[1] JUN SAKURAI,[3] AND RICHARD W. TITBALL[1]*

Chemical and Biological Defence Establishment, Porton Down, Salisbury, Wiltshire SP4 0JQ, United Kingdom[1]; United States Army Medical Research Institute for Infectious Diseases, Fort Detrick, Frederick, Maryland 21702[2]; and Faculty of Pharmaceutical Sciences, Tokushima Bunri University, Tokushima 770, Japan[3]

Oligonucleotide probes designed on the basis of the N-terminal sequence of *Clostridium perfringens* beta-toxin were used to isolate the encoding gene (*cpb*). The nucleotide sequence of *cpb* was determined, and on the basis of DNA hybridization experiments it was shown that the gene is found only in type B and C strains of *C. perfringens*. The deduced amino acid sequence of the beta-toxin revealed homology with the alpha-toxin, gamma-toxin, and leukocidin of *Staphylococcus aureus*. The beta-toxin purified from *C. perfringens* appeared to exist in monomeric and multimeric forms. Recombinant beta-toxin, produced in *Escherichia coli*, appeared to be mainly in the multimeric form.

The etiology of diseases caused by *Clostridium perfringens* can be ascribed almost entirely to the potent exotoxins produced by the bacterium. Of the major toxins, alpha-toxin and epsilon-toxin are associated mainly with disease in humans and animals, respectively (17). In contrast, the beta-toxin is known to play a major role in the pathogenesis of necrotic enteritis in both hosts. In humans, the disease has been termed pigbel (12, 14, 15) and has been reported mainly in Africa, Southeast Asia, and New Guinea. It has been suggested that the disease occurs when the breakdown of beta-toxin, produced by *C. perfringens* in the gut, is prevented by the presence of trypsin-inhibitory compounds in the diet. Such trypsin inhibitors often originate from a diet rich in sweet potatoes. Necrotic enteritis attributed to beta-toxigenic *C. perfringens* has been reported in calves, lambs, and pigs (10, 33). Experimental infections when trypsin inhibitors are present in the diet have been reported in guinea pigs (14), but it is not clear whether the presence of dietary trypsin inhibitors is a prerequisite for disease in other animals.

Despite the significant association of beta-toxin with these diseases, little is known of the biophysical or molecular biological properties of the toxin; the toxin has been isolated in a pure form (21, 23) and shown to be lethal in mice (22) and guinea pigs (23). The mode of action of the toxin has not been elucidated, but the toxin has been shown to be dermonecrotic, to induce hemorrhagic necrosis in guinea pig intestinal loops (14), and to be cytotoxic for Chinese hamster ovary cells (13).

To study this toxin in more detail, we planned to isolate the encoding gene and determine its nucleotide sequence. This approach could facilitate the design of probes for the detection of the encoding gene and, in the longer term, the development of effective prophylactic or therapeutic reagents against beta-toxin-mediated diseases.

MATERIALS AND METHODS

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 addition of Na_2HPO_4 (TPYG broth). For isolation of DNA, *C. perfringens* cells 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. *Escherichia coli* JM101 and JM109 were stored and cultured as described by Sambrook et al. (25).

Preparation of DNA. Chromosomal DNA was isolated from the bacteria used in this study by the method of Marmur (16). DNA extracted from *C. perfringens* type B (NCTC 8533) was purified by centrifugation through a gradient of cesium chloride (25). Large- and small-scale plasmid isolations were performed as described by Sambrook et al. (25). Oligonucleotides were prepared with a Beckman 200A DNA synthesizer with the final trityl group left on. The oligonucleotides were purified with a cartridge purification system (Applied Biosystems).

N-terminal amino acid sequence of beta-toxin. Beta-toxin was purified from *C. perfringens* type C (CN 5386) as described previously (23). The N-terminal amino acid sequence of the toxin was determined independently at the U.S. Army Medical Research Institute for Infectious Diseases and the Faculty of Pharmaceutical Sciences, Tokushima Bunri University, with a gas-phase sequencer (470A; Applied Biosystems) equipped with an on-line phenylthiohydantoin analyzer (120A; Applied Biosystems).

Blotting of DNA and hybridization of gene probes. After electrophoresis, DNA digested with restriction endonucleases was transferred onto a nylon membrane (Hybond-N; Amersham). Undigested DNA was transferred onto the membrane with a slot blot apparatus. Oligonucleotide probes (10 pmol) were 5' end labelled (25). Unincorporated [γ-32P]ATP was removed by passing the labelled oligonucleotide through a Nensorb 20 column [DuPont (UK) Ltd.] in

* Corresponding author.

accordance with the manufacturer's instructions. Isolated fragments of double-stranded linear DNA were labelled with $[\alpha\text{-}^{32}P]dCTP$ by using a random-primed labelling kit (Boehringer Mannheim UK Ltd.). The labelled probes were hybridized to membranes as described by Sambrook et al. (25). For oligonucleotide probing, the temperature of hybridization and washing was 42°C (ClosNH and ClosCOO), 34°C (βtox3), or 40°C (βtox2).

Cloning techniques. Two strategies were used to clone DNA fragments from *C. perfringens* type B (NCTC 8533). Chromosomal DNA digested with restriction endonuclease *Ssp*I was ligated to 0.1 μg of digested and dephosphorylated plasmid pUC18 and transformed into *E. coli* JM101 cells (9). Cells were transferred to a nylon membrane (Hybond-N) by colony blotting as described by Sambrook et al. (25), except that after replica plating, the colonies were incubated on L agar containing chloramphenicol (100 μg/ml) to amplify the plasmid DNA.

Chromosomal DNA digested with *Hin*dIII or *Sau*3A was ligated to suitably digested and dephosphorylated M13mp18 (*Hin*dIII) or M13mp19 (*Sau*3A) (Pharmacia LKB Biotechnology), transfected into *E. coli* XLI-blue (Stratagene), and mixed with *E. coli* JM105 or TG1 cells before inoculation onto plates of L agar plus ampicillin (25). The plaques were transferred onto a nylon membrane (Hybond-N) as described by Sambrook et al. (25).

After incubation of the membranes with radioactively labelled oligonucleotides as described above, colonies or plaques which hybridized with the probe were picked from the original plate and subcultured.

Nucleotide sequencing. Single-stranded DNA was prepared from M13 clones as described by Sambrook et al. (25). To generate single-stranded DNA from plasmid clones, the insert DNA fragments were amplified by using the polymerase chain reaction (PCR) with one of the oligonucleotide primers biotin labelled (Dynal A.S., Oslo, Norway). The biotin-labelled strand of DNA was isolated by using streptavidin-coated magnetic beads (Dynabeads; Dynal A.S.). Nucleotide sequencing was performed by the dideoxy-chain termination method with cloned T7 DNA polymerase (Sequenase Version 2.0; United States Biochemical Corp.) in accordance with the manufacturer's instructions.

Alternatively, nucleotide sequencing of the positive strand from M13 cloned DNA fragments was performed by isolating the M13 replicative form (25), which then served as a template for use in a *Taq* polymerase cycle sequencing system (GIBCO-BRL).

Computer analysis of nucleotide and amino acid sequences. Nucleotide and deduced amino acid sequence data were analyzed with DNAStar computer software. The amino acid sequence of *Staphylococcus aureus* alpha-toxin (8) was translated from the nucleotide sequence located in the EMBL sequence data base under accession no. X01645 and amended by changing the following residues: Gly-25 to Asn, Ala-74 to His, Iso-75 to Asn, and Glu-123 to Gln (13a). The amino acid sequences of beta-toxin and the individual *S. aureus* toxins were compared by using the PROSCAN and AALIGN programs (DNAStar). Multiple sequence alignments and comparisons were done with the Clustal algorithm within the MEGALIGN program (DNAStar). Hydropathy (Hopp-Woods algorithm) and surface exposure (Emini algorithm) predictions were done with the appropriate DNAstar programs.

Expression of beta-toxin in *E. coli*. A DNA fragment which would encode the open reading frame of the beta-toxin and the proposed Shine-Dalgarno sequence was amplified from

C. perfringens NCTC 8533 DNA by using the PCR. Oligonucleotides used in the PCR corresponded to nucleotides 316 to 338, with an additional 5' region that encodes an *Eco*RI site (GTCGACGAATT), and the complementary sequence of nucleotides 1335 to 1355, with an additional 5' region that encodes a *Pst*I site (AAGCTTGGCTGCAG). A DNA fragment was obtained after 35 cycles of amplification (95°C, 15 s; 45°C, 15 s; 72°C, 30 s; Perkin Elmer 9600 GeneAmp PCR System). The fragment was purified, digested with *Eco*RI and *Pst*I, ligated with suitably digested plasmid pUC19, and transformed into *E. coli* JM109 cells as described above. A colony containing the cloned beta-toxin gene (plasmid pBET7) was identified by using the PCR with the oligonucleotides described above. A culture of *E. coli* containing pBET7 was grown in L broth (37°C with shaking at 150 rpm) to an optical density at 600 nm of 0.5, at which time isopropyl-β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 1 mM and the culture was grown for a further 18 h. Periplasmic and cytoplasmic fractions of these cells were prepared as described previously (29).

SDS-PAGE and Western blotting (immunoblotting). Purified beta-toxin (100 μg/ml; 23) or extracts from *E. coli* containing pBET7 were mixed with sample buffer (4% [wt/vol] sodium dodecyl sulfate [SDS], 5% [vol/vol] β-mercaptoethanol), incubated (5 min, 22 or 100°C), and analyzed by SDS-polyacrylamide gel electrophoresis (PAGE) (0.1% SDS). In some experiments, the separated material was transferred onto a polyvinylidene difluoride membrane (Immobilon P; Millipore Corp.) and the membrane was blocked with a 5% (wt/vol) solution of dried skim milk in phosphate-buffered saline. The membrane was probed with a 1:1,000 dilution of a mouse anti-beta-toxin monoclonal antibody (F154/3-G7), and bound antibody was detected with a horseradish peroxidase-labelled anti-mouse immunoglobulin G antibody (Sigma) by visualization of the bound antibody with a 1-chloro-4-naphthol substrate (25). In some experiments, horseradish peroxidase-labelled horse anti-*C. perfringens* type B serum (Wellcome Diagnostics) was used to probe the membrane directly, with visualization of the bound antibody as described above.

Nucleotide sequence accession number. The nucleotide sequence of the beta-toxin gene from *C. perfringens* has been submitted to GenBank and assigned accession no. L13198.

RESULTS

Design of oligonucleotide probes. Four oligonucleotides (ClosNH, ClosCOO, βtox2, and βtox3) were designed on the basis of the experimentally determined N-terminal amino acid sequence of beta-toxin. The probes were designed to be complementary to the coding (βtox2 and βtox3) or noncoding (ClosNH and ClosCOO) strand of the DNA (Fig. 1) and were selected from regions where possible codon degeneracy was minimal (26). To reduce the redundancy of two of the oligonucleotides further, the neutral base analog deoxyinosine (26) was incorporated into probe βtox3 at three positions and into βtox2 at two positions. The probes were also designed with reference to known codon usage preferences in *C. perfringens* (30) and, for ClosNH, ClosCOO, and βtox2, the ability of guanine and thymidine to pair with neutral bias. With these strategies, the probes were reduced to 128-fold (ClosNH), 64-fold (ClosCOO), 4-fold (βtox2), or 12-fold (βtox3) degeneracy.

Hybridization of oligonucleotide probes. DNA from *C. perfringens* type B (NCTC 8533) was digested with *Hin*dIII,

```
A. N   D   I   G   K   T   T   T   I   T   R   N   K   T   S   D   G   Y   T   I   I   T   Q   N   D   K   Q   I   I   S   Y   Q

B. Asn Asp Ile Gly Thr Thr Thr Thr Ile Thr Arg Asn Asn Thr Ser Asp Gly Tyr Thr Ile Ile Thr Gln Asn Asp Lys Gln Ile Ile Ser Tyr Gln

C. AAC GAC AUA GGA ACA ACA ACA ACA AUA ACA GGA AAC AAC ACA UCA GAC GGA UAC ACA AUA AUA ACA CAA AAC GAC AAA CAA AUA AUA UCA UAC CAA
    U   U   C   C   C   C   C   C   C   C   U   U   C   C   U   C   U   C   U   C   C   C   C   G   U   U   G   G   C   C   C   U   G
            U   G   G   G   G   G   U   G   G                   G   G       G       G   U   U   G               U           U   G
            U   U   U   U   U       U   U                       U   U       U       U           U                           U
                            AGA                             AGC                                                         AGC
                            G                               U                                                           U

D. AAT GAT ATA GGA ACA ACA ACA ACA ATA ACA AGA AAT AAT AC
        T   T   T   T   T   T   T

E.                                             GAT GGA TAT ACA ATA ATA ACA CAA AAT GAT AAA CAA ATA AT
                                                    T       T   T   T   T                       T

F.                                             TAI TGI GTT TTA CTA TTT GTT TA
                                                            G   G

G.                                         CTA CCI ATG TGI TAT TAI TG
                                               G       A       G
                                                               A
```
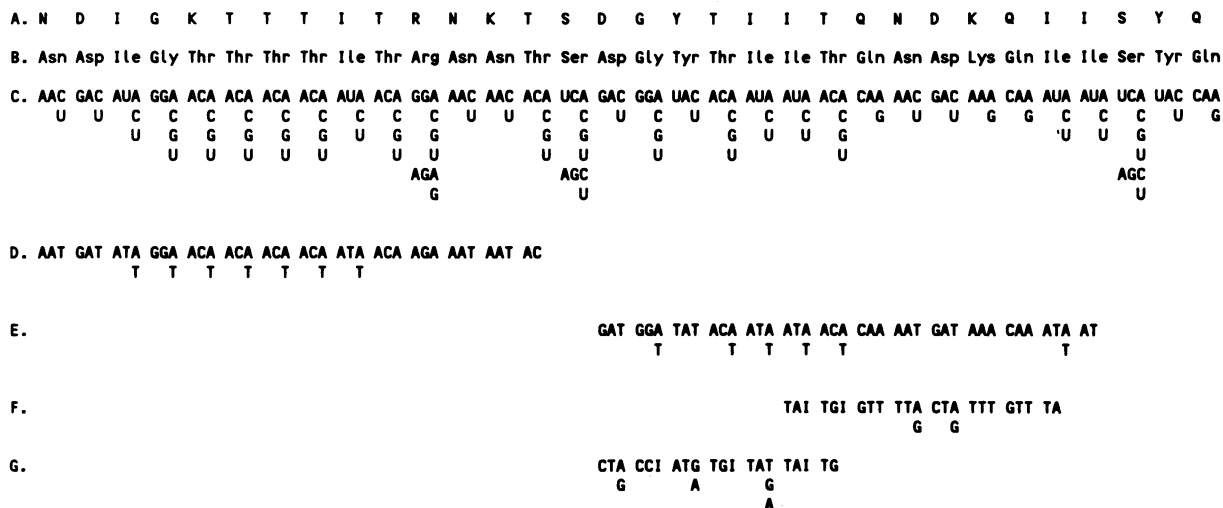
FIG. 1. Experimentally determined N-terminal amino acid sequence of beta-toxin (B), showing the possible mRNA that codes for this sequence (C). Oligonucleotide probes ClosNH (D), ClosCOO (E), βtox2 (F), and βtox3 (G) were designed by taking into account the codon usage bias in *C. perfringens*. To reduce the degeneracy of the βtox2 and βtox3 probes further, the neutral-pairing base analog deoxyinosine was used at position 3 in the codon for Gly, Thr, and Ile. The N-terminal amino acid sequence of beta-toxin deduced from the experimentally determined nucleotide sequence is shown on line A.

*Sau*3A, or *Ssp*I, and fragments which hybridized with βtox2 or βtox3 were identified by Southern blotting. The hybridization temperatures were calculated (26) to be 42°C (probes ClosNH and ClosCOO), 40°C (probe βtox2), and 34°C (probe βtox3). The probes all reacted with a 300-bp *Ssp*I fragment, a 700-bp *Sau*3A fragment, or an 850-bp *Hin*dIII fragment. In some experiments, probes ClosNH and Clos COO reacted with a 1.4-kb *Sau*3A fragment. On the basis of these results, probes ClosNH, ClosCOO, and βtox2 were selected for isolation of the cloned DNA fragments because the higher hybridization temperature would minimize nonspecific probe binding.

Cloning of the beta-toxin-encoding gene. DNA fragments generated after *Ssp*I digestion were cloned into plasmid pUC18, and approximately 5,000 ampicillin-resistant colonies were screened by hybridization with oligonucleotide βtox2. A single probe-reactive colony was identified, the plasmid DNA was isolated (pBET400), and the identity of the DNA insert was confirmed by Southern blotting and reaction with probe βtox2. The nucleotide sequence of the probe-reactive fragment was determined; this revealed a sequence which could encode the N terminus of beta-toxin (Fig. 1A). Two differences between the experimentally determined and deduced amino acid sequences were observed, at positions 5 (threonine-lysine) and 13 (asparagine-lysine).

To isolate fragments of DNA that encode the remainder of the beta-toxin gene, fragments of DNA generated after *Sau*3A or *Hin*dIII digestion were cloned directly into bacteriophage M13. Approximately 10^5 plaques were screened from each library, of which 75 and 46%, respectively, appeared to contain insert DNA, as judged by inactivation of the *lacZ* gene. Twenty-five plaques which reacted with probes ClosNH and ClosCOO were identified, isolated, and plaque purified. Examination of the replicative-form DNA revealed that two of the *Hin*dIII clones (pBET2 and pBET4) contained a 0.85-kb fragment. A single *Sau*3A clone (pBET26), which contained a 1.4-kb DNA insert, was also selected for further characterization.

Nucleotide sequence determination. The nucleotide se-

quences of clones pBET400, pBET2, and pBET26 were determined by using a variety of sequencing strategies (Fig. 2). The nucleotide sequence of the pBET26 DNA insert was not determined at the 3' end. The nucleotide sequences of the three DNA fragments overlapped, and a total of 1,524 bp of the nucleotide sequence was obtained.

Features of the open reading frame. Examination of the nucleotide sequence of the cloned DNA fragments revealed the presence of a single open reading frame between nucleotides 333 and 1340 (Fig. 2). This open reading frame contained regions of DNA homologous with the oligonucleotides designed on the basis of the beta-toxin amino acid sequence. Comparison of the deduced amino acid sequence with the N-terminal amino acid sequence of beta-toxin indicated that amino acid Asn-28 corresponded to the start of the mature protein. The deduced amino acid sequence before this point showed features typical of a signal sequence (32); this region was characterized by a central hydrophobic domain (Fig. 3) and terminated with an alanine residue. Processing of the gene product, on export, would yield a protein with a calculated molecular weight of 34,861. The predicted isoelectric point of *C. perfringens* beta-toxin (5.5) was similar to previously reported values which had been experimentally determined (21, 35).

The region of DNA that encodes the beta-toxin (*cpb*) was A+T rich (71.5% A+T, which is close to the reported composition of *C. perfringens* DNA [73 to 76% A+T] [4]).

A possible Shine-Dalgarno region was located 7 bp upstream of the ATG start codon of the *cpb* gene. This region could form a stable bond with the 3' end of the 16S rRNA from *C. perfringens* ($\Delta G = -20.2$ kcal [1 cal = 4.184 J]). The region between the putative ribosome-binding site and the ATG start codon was composed entirely of thymidine residues. No potential promoter sequences were identified because of the high A+T content of the DNA upstream of *cpb*.

The nucleotide sequence downstream of *cpb* contained no regions predicted to form stable stem-loop structures which were followed by a poly(T) tract, suggesting that rho-independent termination of transcription did not occur.

```
AAGCTTATATTTATGGTGAATTTATGTTGCTCTATTACATTTAGAACATCTAGTTTATCTAGATTGGCGACTGCTCCTGCCTAAGTTAGGAGCAAATTGT   100

CTACCACATTACTTGCATTGATATTTTTTGATTAGCCTGCTTGTCAAATCCAAACTTATATAGTTTTTTTGAATAACAACAAGGACATTTTATATTATTT   200

TTGTTCATTGCTTTTTTCTCCTTTCTTGAGGGAGGATAAATATTTTTTGAAAACTATTGTATTCTCAAGTGTTGAAAAGAGCAATGTTCATTTAACTTAA   300
                  RBS
CAGATCATCTATATACAAAAGGAGGTTTTTTTATGAAGAAAAAATTTATTTCATTAGTTATAGTTAGTTCACTTTTAAACGGATGCCTATTATCACCAAC   400
                              M  K  K  K  F  I  S  L  V  I  V  S  S  L  L  N  G  C  L  L  S  P  T

TTTAGTGTATGCAAATGATATAGGTAAAACTACTACTATAACTAGAAATAAGACATCAGATGGCTATACTATAATTACACAAATGATAAACAGATAATA   500
 L  V  Y  A  N  D  I  G  K  T  T  T  I  T  R  N  K  T  S  D  G  Y  T  I  I  T  Q  N  D  K  Q  I  I

TCATATCAATCTGTTGACTCTTCAAGTAAAAATGAAGATGGTTTTACTGCATCTATAGATGCTAGATTTATCGATGATAAATATTCATCTGAAATGACAA   600
 S  Y  Q  S  V  D  S  S  S  K  N  E  D  G  F  T  A  S  I  D  A  R  F  I  D  D  K  Y  S  S  E  M  T  T

CTTTAATAAACTTAACTGGATTTATGTCTTCAAAAAAAGAAGATGTTATAAAAAAATACAATTTGCATGATGTTACTAATTCTACTGCAATTAATTTTCC   700
 L  I  N  L  T  G  F  M  S  S  K  K  E  D  V  I  K  K  Y  N  L  H  D  V  T  N  S  T  A  I  N  F  P

GGTTAGATACTCGATTTCTATTTTAAATGAAAGTATTAATGAAAATGTAAAAATAGTTGATAGTATTCCTAAAAATACAATTTCTCAAAAAACTGTATCC   800
 V  R  Y  S  I  S  I  L  N  E  S  I  N  E  N  V  K  I  V  D  S  I  P  K  N  T  I  S  Q  K  T  V  S

AATACAATGGGATACAAAATAGGAGGTTCAATTGAAATAGAAGAAAATAAACCTAAAGCTTCAATTGAAAGCGAATATGCTGAATCATCTACAATAGAAT   900
 N  T  M  G  Y  K  I  G  G  S  I  E  I  E  E  N  K  P  K  A  S  I  E  S  E  Y  A  E  S  S  T  I  E  Y

ATGTCCAACCTGATTTTTCTACTATACAGACAGATCATTCAACCTCTAAAGCTTCATGGGATACAAAATTTACAGAAACTACTCGTGGTAATTATAATTT  1000
 V  Q  P  D  F  S  T  I  Q  T  D  H  S  T  S  K  A  S  W  D  T  K  F  T  E  T  T  R  G  N  Y  N  L

AAAATCAAACAACCCTGTATATGGAAATGAAATGTTTATGTACGGAAGATATACTAATGTTCCTGCAACTGAAAATATAATTCCAGATTATCAAATGTCA  1100
 K  S  N  N  P  V  Y  G  N  E  M  F  M  Y  G  R  Y  T  N  V  P  A  T  E  N  I  I  P  D  Y  Q  M  S

AAATTAATAACAGGTGGTTTAAACCCTAATATGTCTGTAGTTCTAACTGCTCCTAATGGTACTGAAGAATCTATAATAAAAGTTAAAATGGAGCGTGAAA  1200
 K  L  I  T  G  G  L  N  P  N  M  S  V  V  L  T  A  P  N  G  T  E  E  S  I  I  K  V  K  M  E  R  E  R

GAAACTGTTATTATCTTAATTGGAATGGTGCTAACTGGGTAGGACAAGTCTATTCCAGGCTAGCTTTTGATACCCCAAATGTAGATAGTCATATATTTAC  1300
 N  C  Y  Y  L  N  W  N  G  A  N  W  V  G  Q  V  Y  S  R  L  A  F  D  T  P  N  V  D  S  H  I  F  T

ATTCAAAATAAATTGGCTTACTCACAAAGTAACAGCTATTTAGACTTTTATATTTGTACTAATATGAATTTCAAATTAGTCCTTCGTATGTAATTTTAAT  1400
 F  K  I  N  W  L  T  H  K  V  T  A  I  *

ACGGAAAGATGTAGCAAATGGTAATGAAGATGGTAGCATAACAAATGAAATTACTATATCTAATAGGGGGAGCGTCCATAGCTGTCAACCTAAGAAGCCT  1500


CACTTTCTATAATTAATTATTATA
```

FIG. 2. Nucleotide sequence of the *C. perfringens* DNA fragment that encodes beta-toxin. The nucleotide sequence of *cpb* is shown between nucleotides 333 and 1340. The deduced amino acid sequence of beta-toxin is shown below the coding sequence. The region which corresponded to the experimentally determined N-terminal sequence of beta-toxin is underlined. Also shown are the proposed ribosome-binding site (RBS) located upstream of the ATG start codon and the TAG stop codon (*).

Expression of *cpb* in *E. coli*. A DNA fragment which would precisely encode the beta-toxin open reading frame and the ribosome-binding site was generated from *C. perfringens* NCTC 8533 DNA by using a PCR with primers which bound upstream and downstream of the gene. The DNA fragment was cloned into plasmid pUC19 to generate plasmid pBET7. IPTG was added to cultures, and the cells were fractionated to determine the location of beta-toxin. The proteins were separated by SDS-PAGE and Western blotted with an anti-beta-toxin monoclonal antibody. A major reactive protein was detected in extracts from the periplasmic space of cells containing plasmid pBET7 but not in cells containing pUC19 alone (Fig. 4). Similar results were obtained when horse polyclonal anti-*C. perfringens* type B serum was used as the primary antibody. The apparent molecular mass of the main protein band was 118 kDa, and some immunoreactive bands with higher apparent molecular masses were also visible. A minor component with an apparent molecular mass of 34 kDa was also visible on Western blots. When beta-toxin purified from *C. perfringens* (23) was analyzed, two protein bands which reacted with the antibody, with sizes similar to those of the *E. coli*-expressed proteins, were detected. The 34-kDa component was the predominant immunoreactive form (Fig. 4). Incubation of the samples in sample buffer, without boiling, did not alter the profile of immunoreactive bands.

Homology of beta-toxin with other proteins. The deduced beta-toxin amino acid sequence was screened against the Swiss-Prot and PIR data bases with DNAStar computer software. We found significant homology with the deduced amino acid sequence of *S. aureus* alpha-toxin (13a) (28% similarity), the A, B, and C components of gamma-toxin (6) (22, 28, and 18% similarity, respectively), and the S and F components of leukocidin (19, 20) (17 and 28% similarity, respectively) and leukocidin S (27) (18 and 29% similarity, respectively) (Fig. 5). All of these proteins were similar in size (286 to 309 amino acids), and alignment of the deduced amino acid sequences revealed that 16 amino acids were identical and 95 were conservatively substituted. The homology between these proteins was weakest at the N termini (to residue 52 in Cpb).

Biophysical properties of beta-toxin and *S. aureus* alpha-toxin. In view of the amino acid sequence homology between *C. perfringens* beta-toxin and *S. aureus* alpha-toxin and the fact that both are single-component toxins, it was of interest to compare the predicted biophysical properties of these proteins. Both proteins were predicted to be composed
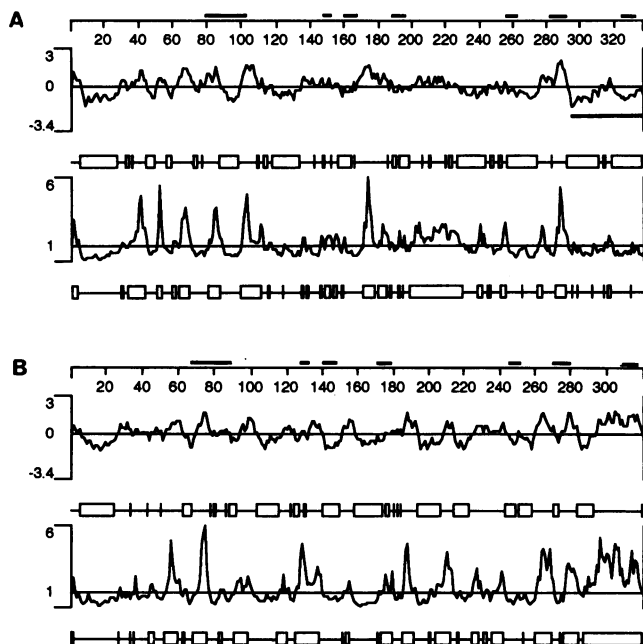
FIG. 3. Predicted hydropathy (upper trace) and surface exposure (lower trace) profiles of the unprocessed forms of *C. perfringens* beta-toxin (A) and *S. aureus* alpha-toxin (B). Positive values within the hydrophilicity plot indicate hydrophilicity, and negative values indicate hydrophobicity. The locations of predicted hydrophobic and surface-exposed regions are also shown boxed below the appropriate traces. The locations of areas of high amino acid sequence homology are indicated above the amino acid numbering. The hydrophobic region present only in beta-toxin is underlined.

mainly of a beta-sheet structure (48 and 49%, respectively), and circular dichroism studies have previously shown that alpha-toxin is composed largely (60%) of a beta-sheet structure (31). Comparison of the hydropathy and surface exposure profiles revealed little overall similarity between these proteins (Fig. 3). To study the structural similarity between these proteins in more detail, the hydropathy and surface exposure profiles within the highly homologous regions of
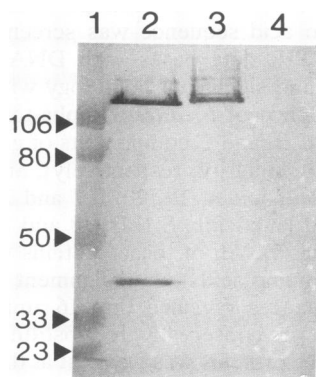

FIG. 4. Analysis of beta-toxin and recombinant beta-toxin by SDS-PAGE and Western blotting. Samples of purified beta-toxin (40 μg/ml; track 2) or extracts from the periplasmic space of *E. coli* containing pBET7 (lane 3) or pUC19 (lane 4) were separated by SDS-PAGE, Western blotted, and reacted with an anti-beta-toxin monoclonal antibody. Prestained molecular size markers (in kilodaltons) were in lane 1.

these proteins were examined more closely and also compared with the profiles of the colocated regions in LukF and HlgB. The N terminus of the region between amino acids 80 to 102, region 146 to 148, and the C terminus of region 281 to 290 in Cpb were predicted to be surface exposed, and the colocated regions within Hla (Fig. 3), LukF, and HlgB (data not shown) were predicted to have similar properties. The region between amino acids 159 and 167 in Cpb and the colocated regions in Hla (Fig. 3), LukF, and HlgB (data not shown) were predicted to be hydrophobic and buried. Other regions of amino acid similarity between Cpb and the *S. aureus* toxins showed little structural similarity. The C termini of the *S. aureus* proteins were all predicted to be hydrophilic. The C terminus of Cbp was hydrophobic and could form a membrane-spanning region.

Probing of clostridial DNA for the beta-toxin gene. DNAs isolated from *C. perfringens* types A, B, C, and D were hybridized with radiolabelled plasmid pBET400 in a slot blot assay. The results (Fig. 6) indicated that the probe hybridized only with DNAs from *C. perfringens* types B and C. In other experiments, the probe failed to hybridize to DNA isolated from *C. botulinum* type B, E, or F; *C. perfringens* type E (CN5063); or *C. novyi* type A (NCTC 538) or D (NCTC 8350 or NCTC 9692) (data not shown).

DISCUSSION

Three degenerate probes designed on the basis of the N-terminal amino acid sequence of purified beta-toxin were used to isolate the beta-toxin gene (*cpb*) from *C. perfringens* NCTC 8533. The deduced N-terminal amino acid sequence of beta-toxin did not match the experimentally determined sequence precisely, indicating that the proteins from two different strains of *C. perfringens* were not identical. The differences observed (Thr-5 to Lys and Asp-13 to Lys) were not conservative substitutions. It is unlikely that these differences were due to errors in the N-terminal amino acid sequence, since this was determined independently in two laboratories. The importance of these residues for the biological activity of the toxin could not be elucidated, since we did not compare the specific toxicities of the proteins. DNA hybridization studies revealed that *cpb* was found only in strains of *C. perfringens* types B and C, and these types are associated with beta-toxin production. It has previously been reported that beta-toxin is plasmid encoded (7), and our method of isolating bacterial DNA would not have discriminated between chromosomal and plasmid species (11).

Beta-toxin is one of the main extracellular products of type B and C strains of *C. perfringens*. Some features of the upstream nucleotide sequence suggested that translation of *cpb* mRNA would be efficient: the possible Shine-Dalgarno sequence, identified upstream of the ATG start codon, would form a highly stable hybrid with the 3' end of the *C. perfringens* 16S rRNA. The intervening seven-nucleotide spacing would be optimal for gene expression (34), and the unusual poly(T) composition could limit secondary structure, thus aiding efficient translation (34). The failure to identify possible −10 and −35 promoter sequences upstream of the ribosome-binding site may be due to the high A+T content of this region. Alternatively, it is possible that the gene forms part of a larger operon, but the location of translational termination signals, in all three reading frames, upstream of *cpb* indicated that the 3' end of an open reading frame would be located at least 300 bp upstream of *cpb*.

Comparison of the start of the beta-toxin N-terminal amino acid sequence with the deduced amino acid sequence

```
Cpb    NDIGKTTTITRNKTSDGYTIITQNDKQIISYQSVDSSSKNEDGFTASIDARFIDDKYSSEHTTLINLTGFMSSKKEDVIKKYNLHDVTNSTAINFPVRYS  100
Hla    AD-SDI---NIKTGTTDIGSNTTVK------TGDLVTYDKENGMHKKVFYSFIDDKNHNKKLLVIRTKGTIAGQ----YRVYSEEGANKSG-LAWPSAFK   85
HlgB   AE-GKITPVSVKKVDDKVTLYKTTA------TADSDKFK----ISQILTFNFIKDKSYDKDTLVLKATGNINSG----FVKPNPNDYDFSK-LYWGAKYN   84
LukF-R -E-GKITPVSVKKVDDKVTLYKTTA------TADSDKFK----ISQILTFNFIKDKSYDKDTLVLKAAGNINSG----YERPNPKDYDFSK-IYWGAKYN   83
LukF   -E-GKITPVSVKKVDDKVTLYKTTA------TADSDKFK----ISQILTFNFIKDKSYDKDTLVLKATGNINSG----FVKPNPNDYDFSK-LYWGAKYN   83
HlgA   EN-KIE---DIGQGA---EIIKRTQ------DITSKRL----AITQNIQFDFVKDKKYNKDALVVKNQGFISSR----TTYSDLKKYPYIKRMIWPFQYN   79
HlgC   AN-DTE---DIGKGSD-IEIIKRTE------DKTSNKW----GVTQNIQFDFVKDKKYNKDALILKNQGFISSR----TTYNYKKTNHVKAMRWPFQYN   81
LukS   AN-DTE---DIGKGSD-IEIIKRTE------DKTSNKW----GVTQNIQFDFVKDTKYNKDALILKNQGFISSR----TTYYNYKKTNHVKAMRWPFQYN   81
LukS-R AN-DTE---DIGKGND-VEIIKRTE------DKTSNKW----GVTQNIQFDFVKDKKYNKDALILKNQGFISSR----TTYYNYKKYNHIKAMRWPFQYN   81
                   .         .              .    *..*.  ..  ... * ...              . .      ,.   ..

Cpb    ISILNESINENVKIVDSIPKNTISQKTVSNTMGYKIGGSIEIEENKPKASIES-EYAESSTIEYVQPDFSTIQTDHSTSKA-SWDTK---FTETTRGNYN  195
Hla    VQLQLPD-NEVAQISDYYPRNSIDTKEYMSTLTYGFNGNVTGDDTGKIGGLIGANVSIGHTLKYVQPDFKTILESPTD-KKVGNKVIFNNMVNQNWGPYD  183
HlgB   VSISSQS-NDSVNVVDYAPKNQNEEFQVQNTLGYTFGGDISISN-GLSGGLNG-NTAFSETINYKQESYRTTLSRNTNYKNVGWGVEAHKIMNNGWGPYG  181
LukF-R VSISSQS-NDSVNVVDYAPKNQNEEFQVQNTLGYTFGGDISISN-GLSGGLNG-NTAFSETINYKQESYRTTLSRNTNYKNVGWGVEAHKIMNNGWGPYG  180
LukF   VSISSQS-NDSVNAVDYAPKNQNEEFQVQNTLGYTFGGDISISN-GLSGGLNG-NTAFSETINYKQESYRT-LSRNTNYKNVGWGVEAHKIMN-GWGPYG  178
HlgA   ISLKT-K-DSNVDLINYLPKNKIDSADVSQKLGYNIGGNFQ-SAPSIGGS---GSFNYSKTISYNQKNYVTEVESQNS-KGVKNGVKANSFVTPN-----  167
HlgC   IGLKT-N-DKYVSLINYLPKNKIESTNVSETLGYNIGGNFQ-SAPSLGGN---GSFNYSKSISYTQQNYVSEVEQQNS-KSVLNGVKANSFATES-----  169
LukS   IGLKT-N-DKYVSLINYLPKNKIESTNVSQTLGYNIGGNFQ-SAPSLGGN---GSFNYSKSISYTQQNYVSEVEQQNS-KSVLNGVKANSFATES-----  169
LukS-R IGLKT-N-DKYVSLINYLPKNKIESTNVSQTLGYNIGGNFQ-SAPSLGGN---GSFNYSKSISYTQQNYVSEVEQQNS-KSVLNGVKANSFATES-----  169
        .  . .  .   *.*  .       ...* ..*.  ..   .      * ...     . *   .

Cpb    LKSNNPVYGNEMFMYGRYTNVPATENIIPDYQMSKLITGGLMPNM--SVVLTAPNGTEESIIKVKNERERN-CYYLNWNGANWVGQVYSRLAFDTPNVDS  292
Hla    RDSWNPVYGNQLFMKTRNGSMKAADNFLDPNKASSLLSSGFSPDFATVITMDRKASKQQTNIDVIYERVRDD-YQLHWTSTNWKGTNT-------KDKWT  275
HlgB   RDSFHPTYGNELFLAGRQSSAYAGQNFIAQHQMPLLSRSNFNPEFLSVLS-HRQDGAKKSKITVTYQREMDL-YQIRWNGFYWAGANY-------KHFKT  272
LukF-R RDSFHPTYGNELFLAGRQSSAYAGQNFIAQHQMPLLSRSNFNPEFLSVLS-HRQDGAKKSKITVTYQREMDL-YQIRWNGFYWAGANY-------KHFKT  271
LukF   RDSFHPTYGNELFLAGRQSSAYAGQNFIAQHQMPLLSRSNFNPRFLSVLS-HRQDAAKKSKITVTYQREMDL-YQIRWNGFYWAGANY-------KHFKT  269
HlgA   --GQVSAYDQYLFAQ-DPTGPAARDYFVPDNQLPPLIQSGFNPSFITTLS-HERGKGDKSEFEITYGRNMDATYAYVTRHR-------LAVDRKHDAFKN  256
HlgC   --GQKSAFDSDLFVGYKPHSKDPRDYFVPDSELPPLVQSGFNPSFIATVS-HEKGSSDTSEFEITYGRNMDVTHAIKRSTHY--GNSYLDGHRVHNAFVN  264
LukS   --GQKSAFDSDLFVGYKPHSKDPRDYFVPDSELPPLVQSGFNPSFIATVS-HEKGSSDTSEFEITYGRNMDVTHAIKRSTHY--GNSYLDGHRVHNAFVN  264
LukS-R --GQKSAFDSDLFVGYKPHSKDPRDYFVPDSELPPLVQSGFNPSFIATVS-HEKGSSDTSEFEITYGRNMDVTHAIKRSTHY--GNSYLDGHRVHNAFKN  264
        . ...   .*      . . . .     .. * ....* .  .    .   . . ...     * ..  .           .

Cpb    HIFT--FKINWLTHKVTAI---------  309
Hla    DRSSERYKIDWEKEEMT----------N  293
HlgB   RTFKSTYEIDWENHKVKLLDTKETENN-  299
LukF-R RTFKSTYEIDWENHKVKLLDTKETENNK  299
LukF   RTFKSTYEIDWENHKVKLLDTKETENNK  297
HlgA   RNVTVKYEVNWKTHEVKIKSITP----K  280
HlgC   RNYTVKYEVNWKTHEIKVKG--Q----N  286
LukS   RNYTVKYEVNWKTHEIKVKG--Q----N  286
LukS-R RNYTVKYEVNWKTHEIKVKG--Q----N  286
        . ....* .....
```

FIG. 5. Alignment of deduced amino acids sequences of C. perfringens beta-toxin and S. aureus gamma-toxin components, leukocidin components, and alpha-toxin. The sequences shown are the processed forms of the following: Cpb, C. perfringens beta-toxin; Hla, S. aureus alpha-toxin (13a); HlgA, HlgB, and HlgC, S. aureus gamma-toxin components A, B, and C (6); LukS (19) and LukS-R (27), slow components of S. aureus leukocidin and leukocidin R; LukF (20) and LukF-R (27), fast components of S. aureus leukocidin and leukocidin R. Identical residues (*) and conservative amino acid substitutions (.) are shown below the aligned sequences.

showed that the first 27 amino acids of the protein could encode a signal sequence directing export of the toxin across the cell membrane. This result is in accordance with the finding that beta-toxin appears in the extracellular fluid in cultures of C. perfringens. When allowance was made for removal of the signal sequence, cpb would encode a protein with a molecular weight of 34,861. Previous workers have found that the protein had apparent molecular masses of 20 to 42 kDa, as determined by gel filtration chromatography or SDS-PAGE (22, 23, 35). In this study, we found that beta-toxin purified from C. perfringens existed in two forms when analyzed by SDS-PAGE and Western blotting: a form with a molecular mass of 34 kDa appears to correspond to the monomeric gene product, and the high-molecular-mass

FIG. 6. Hybridization of recombinant plasmid pBET400, which encodes part of the beta-toxin gene, with purified pBET400 (A1) and DNA isolated from C. perfringens type A (A2, NCTC 8246; A3, NCTC 8237), type B (A4, NCTC 8533; A5, NCTC 6121), type C (B1, NCTC 10719; B2, NCTC 3180; B3, CN 883), type D (B4, NCTC 8346; B5, NCTC 8503), or type E (B6, CN5063).

form of the toxin (118 kDa) may be due to oligomerization of the toxin. The calculated molecular weight does not correspond to a divisible number of subunits, but this may be due to the anomalous migration of the oligomer in SDS-PAGE gels. Beta-toxin isolated from the periplasmic space of E. coli appeared to be mainly in the high-molecular-weight form, and this may be due to the difference in the physiochemical environments of the periplasmic space in E. coli and the culture extracellular fluid of C. perfringens.

Previous workers have reported amino acid sequence similarity between gamma-toxin and alpha-toxin (6) and gamma-toxin and leukocidin or leukocidin R (27). The sequence alignment presented here is the first that compares all members of this family of proteins, and this will aid future studies with these toxins. On the basis of comparisons of amino acid sequences of peptides derived from S. aureus Panton-Valentine leukocidin (27), it seems likely that the F and S components of this toxin also belong to this family of proteins. Several regions within the proteins showed high degrees of amino acid homology and were predicted to be surface exposed, suggesting that they are functionally essential. Identical amino acids located within these regions would be candidates for future investigations of structure-function studies with these toxins. Treatment of beta-toxin with thiol-modifying or oxidizing agents has been reported to abolish lethal activity (24), and as there is a single cysteine residue in the mature protein this is a candidate residue for future
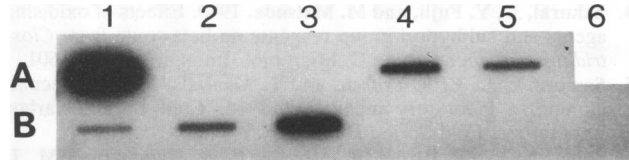
structure-function studies. Several studies have been undertaken to modify essential amino acids in *S. aureus* alpha-toxin chemically; arginine, lysine, and histidine residues appear to be necessary for biological activity (1), but it is not possible to draw conclusions concerning the location or significance of these residues in beta-toxin from the comparison of the deduced amino acid sequences. It has been proposed that *S. aureus* alpha-toxin is composed of two domains separated by a glycine-rich hinge region (1). A glycine-rich region is present in all of the toxins (positions 133 to 138 in Cpb), suggesting that this region performs the same function in all of these proteins. The C-terminal region of alpha-toxin has been suggested to be involved in cell binding (2, 3), and although this region was predicted to be hydrophilic, it is of interest that the C terminus of beta-toxin was predicted to be hydrophobic and potentially membrane spanning.

Both beta-toxin and alpha-toxin are considered to be single-component toxins, whereas gamma-toxin and leukocidin require two separate components to elicit cytolytic activity. However, alpha-toxin can form membrane-active hexamers (18) and in this form it may be appropriate to consider it a multicomponent toxin in which all of the subunits are identical. It is not clear whether the monomeric and oligomeric forms of beta-toxin are toxic, and further studies are required to determine the biological properties and relationship of the different molecular forms.

The cytolytic activity of *S. aureus* alpha-toxin, gamma-toxin, and leukocidin S against a variety of cell types is well characterized (1, 5, 27, 35), and alpha-toxin and gamma-toxin have been classified as pore-forming cytolysins (28). A crude preparation of beta-toxin isolated from the periplasmic space of *E. coli* expressing *cpb* was not hemolytic for rabbit erythrocytes (data not shown), and Jolivet-Raynaud et al. (13) have reported that beta-toxin was cytotoxic but not cytolytic for Chinese hamster ovary cells and inactive against a variety of other cell types. However, some similarities between the toxic properties of alpha-toxin and beta-toxin are apparent from other reports. Fifty percent lethal doses of 1.87 and 0.68 μg, respectively, for mice have been reported (22, 36), and it has been suggested that both toxins elicit effects on the central nervous system (1, 13, 17, 35).

ACKNOWLEDGMENT

REFERENCES

1. **Bhakdi, S., and J. Tranum-Jensen.** 1991. Alpha-toxin of *Staphylococcus aureus*. Microbiol. Rev. **55:**733–751.
2. **Blomquist, L., and A. Sjøgren.** 1988. Production and characterisation of monoclonal antibodies against *S. aureus* alpha-toxin. Toxicon **26:**265–273.
3. **Blomquist, L., and M. Thelestam.** 1986. A staphylococcal α-toxin fragment; its characterisation and use for mapping biologically active regions of α-toxin. Acta Pathol. Microbiol. Immunol. Scand. Sect. B **94:**277–283.
4. **Buchanan, R. E., and N. E. Gibbons.** 1975. Bergey's manual of determinative bacteriology, p. 551–573. The Williams & Wilkins Co., Baltimore.
5. **Clyne, M., T. H. Birkbeck, and J. P. Arbuthnott.** 1992. Characterisation of staphylococcal gamma-lysin. J. Gen. Microbiol. **138:**923–930.
6. **Cooney, J., Z. Kienle, T. J. Foster, and P. W. O'Toole.** 1993. The gamma-hemolysin locus of *Staphylococcus aureus* comprises three linked genes, two of which are identical to the genes for the F and S components of leukocidin. Infect. Immun. **61:**768–771.
7. **Duncan, C. L., E. A. Rokos, C. M. Christenson, and J. I. Rood.** 1978. Multiple plasmids in different toxigenic types of *Clostridium perfringens*: possible control of beta-toxin production, p. 246–248. *In* D. Schlessinger (ed.), Microbiology—1978. American Society for Microbiology, Washington, D.C.
8. **Gray, G. S., and M. Kehoe.** 1984. Primary sequence of the α-toxin gene from *Staphylococcus aureus* Wood 46. Infect. Immun. **46:**615–618.
9. **Hanahan, D.** 1985. Techniques for the transformation of *E. coli*, p. 109–135. *In* D. M. Glover (ed.), DNA cloning: a practical approach, vol. 1. IRL Press, Oxford.
10. **Hauschild, A. H. W.** 1971. *Clostridium perfringens* types B, C, D and E, p. 159–188. *In* S. Kadis, T. C. Montie, and S. J. Ajl (ed.), Microbial toxins. Academic Press, Inc., New York.
11. **Hunter, S. E. C., I. N. Clarke, D. C. Kelly, and R. W. Titball.** 1992. Cloning and nucleotide sequencing of the *Clostridium perfringens* epsilon-toxin gene and its expression in *Escherichia coli*. Infect. Immun. **60:**102–110.
12. **Johnson, S., P. Echeverria, D. N. Taylor, S. R. Paul, R. Conninx, J. Sakurai, B. Eampokalap, P. Jimakorn, R. A. Cooke, G. W. Lawrence, and P. D. Walker.** 1987. Enteritis necroticans among Khmer children at an evacuation site in Thailand. Lancet ii:496–500.
13. **Jolivet-Reynaud, C., M. R. Popoff, M.-A. Vinit, P. Ravisse, H. Moreau, and J. E. Alouf.** 1986. Enteropathogenicity of *Clostridium perfringens* β-toxin and other clostridial toxins. Zentralbl. Bacteriol. Mikrobiol. Hyg. Suppl. **15:**145–151.
13a.**Kehoe, M.** Personal communication.
14. **Lawrence, G., and R. Cook.** 1980. Experimental pig-bel: the production and pathology of necrotizing enteritis due to *Clostridium welchii* type C in guinea pigs. Br. J. Exp. Pathol. **61:**261–271.
15. **Lawrence, G., and P. D. Walker.** 1976. Pathogenesis of enteritis necroticans in Papua New Guinea. Lancet i:125–126.
16. **Marmur, J.** 1961. A procedure for the isolation of deoxyribonucleic acid from micro-organisms. J. Mol. Biol. **3:**208–218.
17. **McDonel, J. L.** 1986. Toxins of *Clostridium perfringens* types A, B, C, D and E, p. 477–517. *In* F. Dorner and J. Drews (ed.), Pharmacology of bacterial toxins. Pergamon Press, Oxford.
18. **McNiven, A., P. Owen, and J. P. Arbuthnott.** 1972. Multiple forms of staphylococcal alpha-toxin. J. Med. Microbiol. **5:**113–122.
19. **Rahman, A., K. Isaki, I. Kato, and Y. Kamio.** 1991. Nucleotide sequence of leukocidin S-component gene (*lukS*) from methicillin-resistant *Staphylococcus aureus*. Biochem. Biophys. Res. Commun. **181:**138–144.
20. **Rahman, A., H. Nariya, K. Isaki, I. Kato, and Y. Kamio.** 1992. Molecular cloning and nucleotide sequence of leukocidin F-component gene (*lukF*) from methicillin-resistant *Staphylococcus aureus*. Biochem. Biophys. Res. Commun. **184:**640–646.
21. **Sakurai, J., and C. L. Duncan.** 1977. Purification of beta-toxin from *Clostridium perfringens* type C. Infect. Immun. **18:**741–745.
22. **Sakurai, J., and C. L. Duncan.** 1978. Some properties of beta-toxin produced by *Clostridium perfringens* type C. Infect. Immun. **21:**678–680.
23. **Sakurai, J., and Y. Fujii.** 1987. Purification and characterisation of *Clostridium perfringens* beta toxin. Toxicon **25:**1301–1310.
24. **Sakurai, J., Y. Fujii, and M. Matsuda.** 1980. Effects of oxidising agents and sulfhydryl group reagents on beta toxin from *Clostridium perfringens* type C. Microbiol. Immunol. **24:**595–601.
25. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
26. **Suggs, S. V., T. Hirose, T. Miyake, E. H. Kawashima, M. J. Johnson, K. Itakura, and R. B. Wallace.** 1981. Use of synthetic oligodeoxyribonucleotides for the isolation of specific cloned DNA sequences. UCLA Symp. Mol. Cell. Biol. **23:**683–693.
27. **Supersac, G., G. Prevost, and Y. Piemont.** 1993. Sequencing of leukocidin R from *Staphylococcus aureus* P83 suggests that staphylococcal leucocidins and gamma-hemolysin are members

of a single, two-component family of toxins. Infect. Immun. **61**:580–587.

28. **Thelestam, M., and R. Mollby.** 1979. Classification of microbial, plant and animal cytolysins based on their membrane damaging effects on human fibroblasts. Biochim. Biophys. Acta **557**:156–169.

29. **Titball, R. W., S. E. C. Hunter, K. L. Martin, B. C. Morris, A. D. Shuttleworth, T. Rubidge, D. W. Anderson, and D. C. Kelly.** 1989. Molecular cloning and nucleotide sequence of the alpha-toxin (phospholipase C) of *Clostridium perfringens*. Infect. Immun. **57**:367–376.

30. **Titball, R. W., H. Yeoman, and S. E. C. Hunter.** 1992. Gene cloning and organisation of the alpha-toxin of *Clostridium perfringens*, p. 211–226. *In* M. Sebald (ed.), Genetics and molecular biology of anaerobic bacteria. Springer-Verlag, New York.

31. **Tobkes, N., B. A. Wallace, and H. Bayley.** 1985. Secondary structure and assembly mechanism of an oligomeric channel protein. Biochemistry **24**:1915–1920.

32. **von Heijne, G.** 1984. How signal sequences maintain cleavage specificity. J. Mol. Biol. **173**:243–251.

33. **Willis, T. A.** 1977. Anaerobic bacteriology: clinical and laboratory practice, 3rd ed. Butterworths, London.

34. **Winnacker, E.-L.** 1987. From genes to clones. VCH Verlagsgesellschaft, Weinheim, Germany.

35. **Wiseman, G. M.** 1975. The hemolysins of *Staphylococcus aureus*. Bacteriol. Rev. **39**:317–344.

36. **Worthington, R. W., and M. S. G. Mulders.** 1975. The partial purification of *Clostridium perfringens* beta-toxin. Onderstepoort J. Vet. Res. **42**:91–98.