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# Cloning, Nucleotide Sequencing, and Expression of the *Clostridium perfringens* Enterotoxin Gene in *Escherichia coli*

JOHN R. CZECZULIN, PHILIP C. HANNA,† AND BRUCE A. McCLANE*

*Department of Molecular Genetics and Biochemistry, University of Pittsburgh School of Medicine, Pittsburgh, Pennsylvania 15261-2072*

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**A complete copy of the gene (*cpe*) encoding *Clostridium perfringens* enterotoxin (CPE), an important virulence factor involved in *C. perfringens* food poisoning and other gastrointestinal illnesses, has been cloned, sequenced, and expressed in *Escherichia coli*. The *cpe* gene was shown to encode a 319-amino-acid polypeptide with a deduced molecular weight of 35,317. There was no consensus sequence for a typical signal peptide present in the 5′ region of *cpe*. Cell lysates from recombinant *cpe*-positive *E. coli* were shown by quantitative immunoblot analysis to contain moderate amounts of CPE, and this recombinant CPE was equal to native CPE in cytotoxicity for mammalian Vero cells. CPE expression in recombinant *E. coli* appeared to be largely driven from a clostridial promoter. Immunoblot analysis also demonstrated very low levels of CPE in vegetative cell lysates of enterotoxin-positive *C. perfringens*. However, when the same *C. perfringens* strain was induced to sporulate, much stronger CPE expression was detected in these sporulating cells than in either vegetative *C. perfringens* cells or recombinant *E. coli*. Collectively, these results strongly suggest that sporulation is not essential for *cpe* expression, but sporulation does facilitate high-level *cpe* expression.**

*Clostridium perfringens* ranks among the most important of the anaerobic bacterial pathogens for humans and domestic animals, causing myonecrosis (gas gangrene), anaerobic cellulitis, septicemia, uterine infections, and gastrointestinal illnesses (28). The virulence of this endospore-forming, gram-positive bacterium results from its prolific ability to produce protein toxins (28). Among the 13 types of toxins known to be produced by this organism is *C. perfringens* enterotoxin (CPE), which is a single polypeptide of 35 kDa with a unique amino acid sequence (9, 23, 24, 28). CPE causes the symptoms associated with *C. perfringens* type A food poisoning, which is among the most common human food-borne illnesses (23, 24, 28), and it also appears to be involved in other important human and veterinary gastrointestinal illnesses (2, 5, 24).

Recent studies (11, 15, 26, 27, 43) have shown CPE action to involve sequentially (i) specific binding of enterotoxin to a proteinaceous receptor on mammalian membranes, (ii) insertion of CPE into plasma membranes, (iii) formation of a complex between enterotoxin and two mammalian membrane proteins of 70 and 50 kDa, (iv) production of membrane permeability alterations for small molecules such as ions, and (v) induction of secondary effects which culminate in cell death. CPE binding to mammalian receptors is mediated by a 30-amino-acid region at the extreme C terminus of enterotoxin (12, 14). Cytotoxicity also requires sequences in the N-terminal half of the enterotoxin, suggesting the existence of separate binding and cytotoxicity domains on CPE (13).

Upon entering the human host, it is common for many bacterial pathogens to undergo global changes in gene expression (30). These changes often include de novo synthesis of virulence factors, including protein toxins (30). During food poisoning, *C. perfringens* demonstrates an unusual variation on this theme. After ingestion in contaminated food, *C. perfringens* encounters the harsh environment of the stomach and small intestine and responds by undergoing changes in gene expression which lead to sporulation (23, 24, 28). Sporulating cells then produce large quantities of enterotoxin, which leads to disease (23, 24, 28).

Although CPE synthesis represents an interesting and biomedically significant model system for studying the regulation of bacterial virulence factor expression, surprisingly little is currently known about the regulation of CPE expression at the molecular level. One of the more significant limitations to detailed molecular studies of CPE synthesis has been the unavailability of a cloned copy of the intact *cpe* gene. At least three independent laboratories have reported the cloning of partial *cpe* fragments in *Escherichia coli* (14, 17, 41). Van Damme-Jongsten et al. obtained overlapping *C. perfringens* DNA fragments which together span the entire *cpe* gene (41). Hanna et al. (14) reported the isolation of a *C. perfringens* DNA fragment encoding the 3′ half of *cpe*. Iwanejko et al. isolated a partial *cpe* fragment which lacked the 3′ region (17). A recent review (9) indicates that since their original report (17), Iwanejko et al. have devoted an extensive research effort to obtaining a complete *cpe* clone. However, despite the utilization of several different cloning strategies, their efforts have continued to be unsuccessful. Given the numerous attempts by several laboratories utilizing independent cloning approaches to clone the complete *cpe* gene, the collective failure of these attempts has led Granum and Stewart to propose recently that cloning the intact *cpe* gene into *E. coli* is highly unfavorable (9).

Contrary to this conjecture, the current study now reports the relatively straightforward cloning of an intact copy of *cpe* into *E. coli*. The recombinant *E. coli* clones carrying *cpe* were found to express moderate amounts of fully cytotoxic CPE, apparently from a clostridial promoter. When combined with Western blot (immunoblot) results comparing CPE expression by sporulating cultures with that by vegetative *C. perfringens* cultures, the *cpe* expression results support the hypothesis that sporulation is not essential for

* Corresponding author.
† Present address: Department of Microbiology and Molecular Genetics, Harvard Medical School, Boston, MA 02115.

CPE expression, but conditions in a sporulating cell do strongly facilitate high-level CPE expression.

## MATERIALS AND METHODS

**Bacterial strains and vectors.** *C. perfringens* NCTC 8239 (Hobbs serotype 3), which is a strong CPE producer (25), was used as the DNA source for cloning the *cpe* gene. *C. perfringens* ATCC 3624, which does not make CPE (25), was used as a negative control strain throughout these studies. The *E. coli* strain DH5α (Bethesda Research Laboratories, Inc., Gaithersburg, Md.) containing the pUC19 vector (45) was used for all cloning experiments. Clones were grown in LB broth or on LB agar, both containing 100 μg of ampicillin per ml.

**Isolation of DNA.** Chromosomal DNA was isolated from *C. perfringens* NCTC 8239 by the method of Marmur (22) except that 2.5% Sarkosyl was substituted for sodium dodecyl sulfate (SDS) to lyse the cells. Plasmid DNA was isolated from clones by the alkali-lysis method of Birnboim and Doly (1).

**Hybridization of *cpe* gene probe.** A previously cloned 2.3-kb *C. perfringens* chromosomal DNA fragment containing the 0.5-kb 3′ half of the *cpe* gene (12, 14) was used as a probe for colony blot hybridization and Southern analysis. The DNA probe was labeled with [α-$^{32}$P]dATP by using a random-primed labeling kit (Bethesda Research Laboratories). The labeled probe was hybridized to membranes as described by Sambrook et al. (34).

**Cloning of *cpe* gene.** The restriction enzyme *Xba*I was used to digest 1 μg of *C. perfringens* NCTC 8239 DNA. The resulting *Xba*I fragments were separated by 0.8% agarose gel electrophoresis and analyzed by Southern blot (37), utilizing the 0.5-kb 3′ *cpe* fragment described above as a probe. Since Southern analysis showed hybridization of the probe to a single band at 5.7 kb (see Results), 100 μg of NCTC 8239 chromosomal DNA was digested with *Xba*I. These *Xba*I-digested DNA fragments were separated by preparative agarose electrophoresis, and bands from 4 to 7 kb were excised and electroeluted. After phenol-chloroform purification, this DNA was ligated into dephosphorylated, *Xba*I-digested pUC19. The recombinant plasmids were then transformed into *E. coli* DH5α. The resulting transformants were subjected to colony hybridization analysis with the 0.5-kb *cpe* probe. All DNA methods used were those of the manufacturer or Sambrook et al. (34).

**DNA sequencing.** The chain termination method of Sanger et al. (35) was used to determine the DNA sequence of the *cpe* gene. Specifically, a Sequenase kit (U.S. Biochemicals, Cleveland, Ohio) was used as described in the manufacturer's specifications. Sequencing primers were constructed to both DNA strands from the consensus *cpe* sequences (12, 17, 41) and then used in double-stranded sequence reactions with plasmid DNA isolated from the recombinant *E. coli* clones which hybridized with the 0.5-kb *cpe* probe.

**Growth of bacteria to test for expression of *cpe* gene.** Recombinant *E. coli* clones were incubated in M9 broth (as modified by Tso and Siebel [40]) for either 6 h or overnight at 37°C with shaking. *C. perfringens* NCTC 8239 (*cpe* positive) and *C. perfringens* ATCC 3624 (which is *cpe* negative [6]) were grown in fluid thioglycolate broth for 8 h at 37°C without shaking to obtain vegetative cultures. To induce sporulation, each *C. perfringens* strain was grown in Duncan-Strong broth (29) for 8 h at 37°C without shaking. To determine if heat-resistant endospores were present in *C. perfringens* cultures, a 1-ml aliquot of vegetative or sporulating *C. perfringens* NCTC 8239 culture was heat shocked at 85°C for 20 min as described previously (7, 10). Dilutions of the heat-shocked culture were plated on brain heart infusion agar and incubated for 16 h at 37°C in GasPak jars. Sporulation in *C. perfringens* cultures was also assessed by phase-contrast microscopy (Nikon, Garden City, N.Y.), where the number of phase-refractile spores per 1,000 bacteria was determined.

**Processing of bacterial cultures to obtain samples for Western blot analysis.** Except for the *E. coli* subcellular fractionation studies described below, all samples for Western analysis of CPE expression were prepared by the following protocol. All bacterial cultures were harvested by centrifugation, and the culture supernatants were removed, concentrated 250-fold by Amicon PM-10 ultrafiltration, and saved for Western blot analysis (see below). Cell pellets were washed twice with TES (10 mM Tris [pH 8.0], 5 mM EDTA, 10 mM NaCl) and lysed by sonication. Unlysed cells and debris were removed by centrifugation, and then lysate supernatants were concentrated 100-fold by Amicon PM-10 ultrafiltration. The protein concentration of lysates were determined with the Bio-Rad protein assay reagent.

**Subcellular fractionation analysis of CPE expression in *E. coli*.** To determine the subcellular location of CPE within *E. coli*, standard fractionation procedures were used (33). Briefly, 6-h cultures of *cpe*-positive recombinant *E. coli* were harvested by centrifugation. The supernatant was removed for 250-fold concentration by Amicon PM-10 ultrafiltration; Western analysis (described below) to examine potential extracellular CPE secretion by *E. coli* followed. The remaining cell pellets were washed twice with 0.01 M Tris-HCl containing 0.03 M NaCl and then resuspended in 20% sucrose containing 0.03 M Tris-HCl (pH 7.3) at room temperature. EDTA was added to a concentration of 0.1 mM, and the suspension was gently shaken for 5 to 10 min. This mixture was then centrifuged (13,000 × *g* for 10 min at 4°C), and the pellet was resuspended in cold 0.5 mM $MgCl_2$. After centrifugation (as described above), the supernatant fluid represented the periplasmic fraction. The remaining pellet was then sonicated to obtain the cytoplasmic fraction.

**Western Blot analysis.** Bacterial lysates were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) as described by Laemmli (19), except that the stacking gel contained 1% SDS. After electrophoresis, proteins were electrotransferred to nitrocellulose membranes (Immobilon-NC transfer membrane; Millipore Corp., Bedford, Mass.) by the method of Towbin et al. (39). These membranes then were blocked with BLOTTO overnight (12, 13) and reacted for 2 h at room temperature with 1 μg of either rabbit anti-CPE immunoglobulin G (IgG) or normal rabbit IgG, both $^{125}$I labeled; per ml. IgG was purified from rabbit anti-CPE serum or normal serum by using an Immunopure IgG purification kit (Pierce Chemical Co., Rockford, Ill.) as described in the supplier's instructions. Purified IgG samples (5 mg) were then radioiodinated as described previously (44) with 1 mCi of Na$^{125}$I (17 Ci/mg; ICN Pharmaceuticals, Inc., Costa Mesa, Calif.).

**Assay for Vero cell cytotoxicity.** Confluent cultures of Vero (African green monkey kidney) cells were prepared and radiolabeled with $^{86}$RbCl (3.75 mCi/mg; Dupont, NEN Research Products) as described previously (12). Radiolabeled Vero cell cultures were then treated for 15 min at 37°C with either purified CPE (29) or specified bacterial lysates, dissolved in 2 ml of Hanks balanced salt solution. Before their application to Vero cells, CPE or bacterial lysates were preincubated for 15 min at 37°C in the absence or presence of

50 μg of either purified monoclonal antibody (MAb) 3C9 (an MAb which was prepared against CPE and which neutralizes CPE cytotoxicity [44]) or 10G6 (an MAb which was prepared against CPE and which reacts with, but does not neutralize, CPE [44]).

After treatment of the Vero cell cultures with CPE or bacterial lysates, $^{86}$Rb release from the cultures was measured as described previously (12). Sample (either CPE or lysate)-induced release of $^{86}$Rb was calculated as follows (38): % of maximal release = [(Sample-induced release − spontaneous release)/(Maximal release − spontaneous release)] × 100.

Spontaneous release represents background $^{86}$Rb release without the addition of sample, while maximal release represents total cytoplasmic radioactivity at the time of sample challenge. Maximal release was $3 \times 10^3$ to $4 \times 10^3$ cpm per culture, and spontaneous release after 15 min was $0.5 \times 10^3$ to $0.7 \times 10^3$ cpm per culture.

**Nucleotide sequence accession number.** The nucleotide sequence of the complete *cpe* gene from *C. perfringens* NCTC 8239 has been submitted to GenBank (accession number M98037).

## RESULTS

**Cloning of the *cpe* gene.** Previously published *cpe* sequence data (12, 17, 41) obtained from overlapping *cpe* fragments were used to generate a computer file containing a complete *cpe* consensus sequence. Computer analysis predicted the absence of an *Xba*I site within the *cpe* gene. Southern analysis of *Xba*I-digested DNA from CPE-positive *C. perfringens* NCTC 8239 showed that a probe consisting of a previously cloned 0.5-kb 3′ *cpe* fragment (12, 14) hybridized to a single 5.7-kb DNA fragment (data not shown), suggesting that the intact CPE gene was located on this *Xba*I fragment. When 4- to 7-kb *Xba*I-digested chromosomal DNA fragments from strain NCTC 8239 were ligated into pUC19 and transformed into *E. coli* DH5α, colony hybridization analysis (data not shown) identified two *E. coli* clones which hybridized with the 0.5-kb *cpe* probe. DNA from both clones (designated A2 and C2) were digested with *Xba*I. Southern analysis confirmed that both clones contained clostridial *Xba*I DNA inserts of 5.7 kb which hybridized with the 0.5-kb *cpe* probe (data not shown). No hybridization of this probe was observed with parent DH5α DNA or with clone C1 (a negative control *E. coli* clone which contains a 5.7-kb clostridial DNA insert but which did not show reactivity with the 0.5-kb *cpe* probe by colony hybridization analysis).

**Nucleotide sequence of *cpe*.** By deduction from previous amino acid sequencing results (8) for mature CPE, nucleotide sequence analysis (Fig. 1) was used to confirm that the 5.7-kb insert in clone A2 contained the intact *cpe* gene. The deduced CPE protein encoded by the NCTC 8239 *cpe* gene of clone A2 would be a 319-amino-acid polypeptide of 35,317 Da. Identical sequence results were also obtained with clone C2 (not shown).

A comparison of the A2 sequence shown in Fig. 1 with previous *cpe* sequencing results reveals some similarities but also several important *cpe* sequence differences between results from different laboratories. The 3′ region of the A2 *cpe* sequence matches exactly our previous sequence derived from a partial 3′ *cpe* clone, independently confirming the accuracy of these earlier results (12). However, a comparison of the entire A2 *cpe* sequence with the *cpe* sequence of Van Damme-Jongsten et al. (41), i.e., the only other published complete *cpe* sequence, shows several striking sequence differences even though both studies used *C. perfringens* NCTC 8239 as the source for the *cpe* gene. For example, the A2 sequence indicates that the *cpe* gene from NCTC 8239 encodes an open reading frame (ORF) of 957 nucleotides (specifying a CPE of 319 amino acids) rather than 960 nucleotides (specifying a CPE of 320 amino acids) as reported by Van Damme-Jongsten et al. (41). This difference derives from significant variation in the extreme 3′ *cpe* sequences between these studies. In agreement with our earlier sequencing results using a partial 3′ *cpe* clone (12), the A2 *cpe* sequence detects a TAC sequence for the codon specifying amino acid 310 of CPE. However, Van Damme-Jongsten et al. (41) reported two codons (TCT CAC) at this sequence location. Besides this major variation leading to differences in the predicted size of the CPE polypeptide, there are also eight single-nucleotide base differences between the A2 *cpe* ORF and the *cpe* ORF sequence of Van Damme-Jongsten et al. (Fig. 1). Seven of these nucleotide sequence differences are silent at the amino acid level, but one difference at nucleotide 331 changes the Glu of the results of Van Damme-Jongsten et al. to a Gln in the A2 sequence (Fig. 1). In addition to these sequence differences within the *cpe* ORF, differences from published sequences (41) were also detected in clone A2 for regions upstream and downstream of the *cpe* ORF. When compared with the results of Van Damme-Jongsten et al. (41), clone A2 has one additional T located 93 bases upstream of the first nucleotide in the *cpe* ORF. More significantly, as depicted in Fig. 1, there are very extensive downstream sequence differences between the A2 sequence and the sequence of Van Damme-Jongsten et al. These downstream sequences are of particular interest since they occur in a region with potential secondary structure and possible regulatory function (see discussion below).

Further analysis of the A2 *cpe* sequence and flanking regions has been conducted to examine features such as the possible presence of an N-terminal signal peptide, the AT content of the *cpe* ORF, the sequence and location of potential ribosome binding sites, and the presence of potential downstream secondary structures. Examination of the A2 sequence strongly suggests that the *cpe* gene does not encode a typical N-terminal secretory signal for CPE secretion since (i) the 5′ codons of the *cpe* ORF directly encode the N-terminal amino acids of mature CPE (8), (ii) there is no typical signal peptide consensus sequence in the 5′ codons of the *cpe* ORF, and (iii) there are in-frame termination codons directly upstream of the identified initiation codon. The *cpe* ORF is very AT rich, with a content (71%) similar to the very high overall AT content (73%) of *C. perfringens* DNA. Sequence analysis of *cpe* from clone A2 also confirms earlier observations (17, 41) indicating the presence of a 6-nucleotide sequence (AGGAGA) 14 bases upstream of the initiation codon which has typical clostridial Shine-Dalgarno sequences. This sequence matches exactly the putative ribosome binding site for another clostridial toxin, *Clostridium difficile* toxin B (42). Finally, the downstream region for clone A2 shown in Fig. 1 extends further than any previously available sequence information. Computer modeling (Fold Program [47]) of this A2 downstream region suggests the presence of a putative stem-loop structure (Fig. 1), with a calculated free energy ($\Delta G$) of −17.3 kcal/mol, which is located 36 bp downstream of the 3′ end of the *cpe* ORF. This putative stem-loop structure is followed by an oligo-T tract, suggesting that this region could function as a rho-independent transcription terminator for *cpe* (32, 46). This region

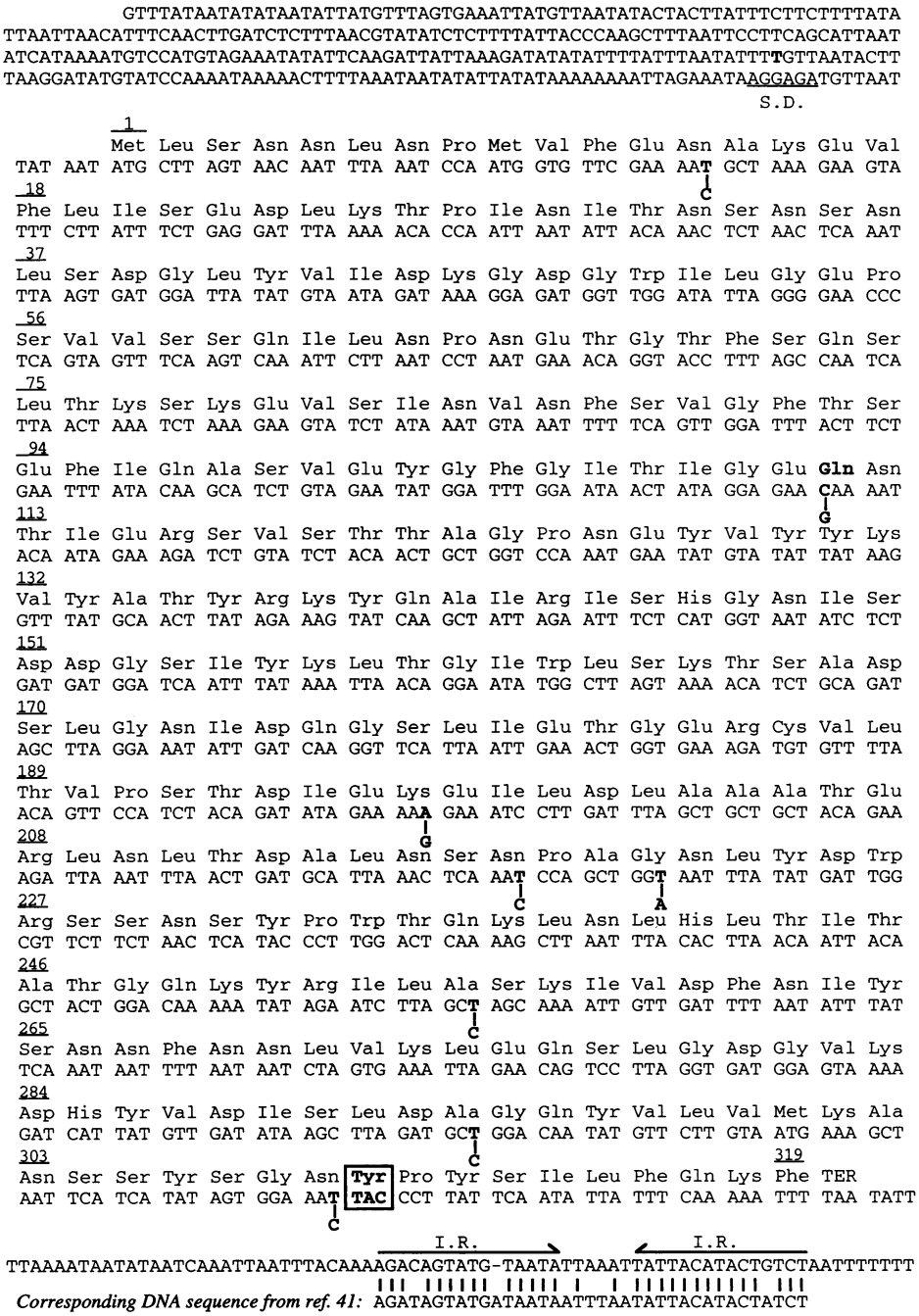


FIG. 1. Nucleotide sequence of the *cpe* gene in clone A2 and the deduced amino acid sequence of *C. perfringens* enterotoxin. The numbers indicate amino acid positions in mature CPE, as deduced by comparison with data generated from amino acid sequencing of native CPE (8). The putative Shine-Dalgarno ribosome binding site is underlined and indicated by S.D. A downstream region containing an inverted repeat which could form a stem-loop structure (see text) is overlined and indicated by I.R. Differences between the A2 sequence and a previous *cpe* sequence generated by Van Damme-Jongsten et al. (41) are marked as follows: (i) silent single-nucleotide base differences in the *cpe* ORF are shown in bold (the sequence of Van Damme-Jongsten et al. is shown below the A2 sequence), (ii) a nucleotide difference specifying a Gln (rather than the Glu of Van Damme-Jongsten et al.) at CPE amino acid 111 is shown in bold, (iii) a difference in the extreme 3′ region of the *cpe* ORF which in clone A2 encodes a Tyr at amino acid 310 of CPE (see text for further discussion) is indicated by bold type contained within a box, (iv) an upstream nucleotide (T) present in the A2 sequence but missing from the sequence of Van Damme-Jongsten et al. is in bold type, and (v) extensive *cpe* downstream differences in the region containing the putative stem-loop structure between the A2 clone sequence and the sequence of Van Damme-Jongsten et al. are aligned for comparison.

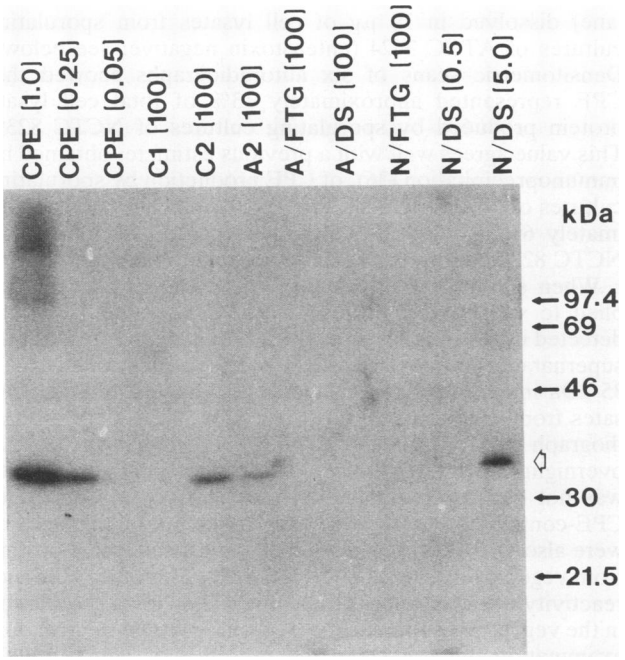


FIG. 2. Western immunoblot analysis of *E. coli* and *C. perfringens* lysates. Lysates from (i) *E. coli* clones that were negative (C1) or positive (C2 and A2) for the *cpe* gene and (ii) *C. perfringens* strains that were negative (ATCC 3624 [abbreviated as A]) or positive (NCTC 8239 [abbreviated as N]) for the *cpe* gene were analyzed by immunoblot analysis for CPE expression by using rabbit anti-CPE [$^{125}$I]IgG as described in Materials and Methods. Quantities of protein (in micrograms) added for each sample are indicated in parentheses. *C. perfringens* strains were grown under both vegetative conditions (in fluid thioglycolate [FTG]) and sporulating conditions (in Duncan-Strong medium [DS]). For comparison, specified concentrations of purified native CPE are included in the three left lanes (the smears at the top of the 1-μg sample of CPE are due to anomalous aggregation which is promoted by high CPE concentrations [27]). The open arrow on the right indicates the migration of purified CPE stained by Coomassie Blue (not shown). No 35-kDa species was present on identical immunoblots developed with normal or preimmune rabbit [$^{125}$I]IgG (data not shown).

could also contribute to the stability of the toxin mRNA transcript (4). This is the first report of possible secondary structure downstream of the *cpe* ORF.

**Expression of *cpe* in *E. coli*.** Once nucleotide sequencing indicated that clones A2 and C2 contained the intact *cpe* gene, Western blot studies were conducted to determine whether CPE could be expressed in *E. coli*. Given the traditional association between CPE expression and sporulation in *C. perfringens* (demonstrated later in Results), the results shown in Fig. 2 are interesting. The Western immunoblot analysis shown in Fig. 2 clearly demonstrates that cell lysates from the *E. coli* A2 and C2 clones which carry the intact *cpe* gene contain a 35-kDa protein which comigrates with purified CPE from *C. perfringens* and specifically reacts with polyclonal antibodies prepared against purified CPE made by *C. perfringens*. This immunoreactive species was absent from cell lysates of *E. coli* control clone C1, which carries a 5.7-kb non-*cpe* clostridial insert. These results demonstrate that the recombinant *E. coli* clones A2 and C2 which carry intact copies of *cpe* can express full-length, mature CPE. The absence of apparent degradative products also indicates that CPE is reasonably stable after expression in *E. coli*.

Subcellular fractionation analysis (33) was conducted to determine the location of CPE within the recombinant *E. coli*. No extracellular enterotoxin was detectable in *E. coli* supernatants by Western analysis (sensitivity limit, <50 ng of CPE), even after culture supernatants were concentrated 250-fold by Amicon ultrafiltration and maximal sample volumes (100 μl) were loaded onto SDS-polyacrylamide gels (data not shown). Given that CPE is not secreted from *C. perfringens* prior to cell lysis (see below) and that CPE appears to lack a typical N-terminal signal peptide, it was somewhat surprising that CPE was detected in both cytoplasmic and periplasmic fractions of log-phase recombinant *E. coli*, at a ratio of 1:2, respectively. The presence of CPE in these periplasmic fractions was not due to a poor fractionation technique since 97% of malate dehydrogenase (a cytoplasmic marker [20]) activity was still present in cytoplasmic fractions of the recombinant *E. coli*.

**Evidence that CPE expression in *E. coli* is driven from a clostridial promoter.** Since both clones A2 and C2 contain the 5.7-kb *Xba*I fragment inserted in the same orientation (data not shown), it was conceivable that CPE expression was being driven from vector sequences rather than clostridial sequences. To examine whether CPE expression in the recombinant *E. coli* clones was being driven from clostridial or pUC19 sequences, the 5.7-kb *Xba*I fragment containing *cpe* was excised from clone C2 and religated in the reverse orientation. The amounts of CPE produced by clones containing the *Xba*I fragment in different orientations were compared by Western analysis (Fig. 3). In three repetitions of Western analysis of CPE expression by two clones containing the *Xba*I fragment in opposite orientations, the amounts of CPE made by the parent and reverse clones were found to be approximately similar. Densitometry of these Western blots indicated that the C2 parent *E. coli* clone produced 1.15 ± 0.39 μg of CPE per mg of cell lysate protein compared to the C2 reverse *E. coli* clone which expressed 0.90 ± 0.21 μg of CPE per mg of cell lysate protein. These results suggest that most, if not all, CPE synthesis observed in the recombinant *E. coli* clones is being driven from a clostridial promoter rather than a plasmid promoter.

**Comparison of CPE expression in *E. coli* and *C. perfringens*.** Once it became apparent that *E. coli* could express CPE, it then became interesting to compare the relative amounts of recombinant CPE made by *E. coli* with the amounts of CPE made by sporulating or vegetative cultures of the NCTC 8239 strain of *C. perfringens* used as the source for the *cpe* cloned into *E. coli*. To address the question of relative expression of CPE by *E. coli* versus that by *C. perfringens*, quantitative Western analysis was conducted.

The amounts of CPE produced by *E. coli* clones A2 and C2 were determined by comparing CPE levels in 100 μg of A2 or C2 lysate with a standard curve derived by using purified CPE (standards ranged from 50 ng to 1 μg of CPE) dissolved in 100 μg of C1 lysate to correct for possible lysate interference. Densitometric scans of autoradiographs from eight repetitions of Western analysis of A2 and C2 lysates (100 or 200 μg of cell lysate protein per sample) showed that CPE represents approximately 0.06 and 0.1% of total cell lysate protein produced by 6-h log-phase cultures of A2 and C2, respectively. This corresponds to approximately 0.2 to 0.4 μg of CPE produced per ml of *E. coli* culture. Overnight cultures of both *E. coli* clones produced about twofold more CPE than log-phase cultures. CPE represented 0.2% of total cell lysate protein produced by overnight cultures of clone

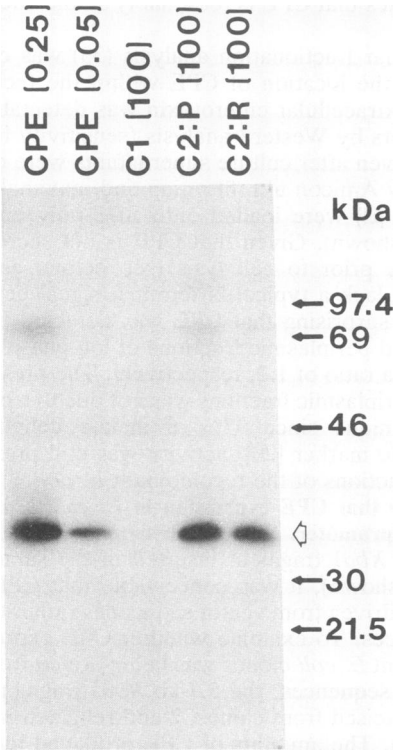


FIG. 3. Western blot evidence that CPE is expressed from a clostridial promoter in recombinant *E. coli*. The 5.7-kb *cpe*-containing fragment in parental *E. coli* clone C2 (C2:P) was excised, religated in the reverse orientation into pUC19, and transformed back into *E. coli* DH5α to create clone C2:R. Lysates from *E. coli* clones that were negative (C1) or positive (C2:P and C2:R) for the *cpe* gene were analyzed by immunoblot analysis for CPE expression by using anti-CPE [$^{125}$I]IgG as described in Materials and Methods. Quantities (in micrograms) of lysate protein added for each sample are indicated in parentheses. For comparison, specified concentrations of purified native CPE are shown in the two left lanes. The open arrow on the right indicates the migration of purified CPE. No 35-kDa species was present on identical immunoblots developed with normal or preimmune rabbit [$^{125}$I]IgG (data not shown).

C2 and 0.1% of total cell lysate protein produced by overnight cultures of A2. This corresponds to 1.3 to 2.7 μg of CPE produced per ml of overnight *E. coli* culture. The reason for the consistent twofold differences in CPE expression between these clones is not apparent, but it should be noted again that restriction analysis (data not shown) indicates that both the A2 and C2 clones contain a single 5.7-kb *Xba*I insert in the same orientation.

For comparative purposes, the expression of CPE by both sporulating and vegetative cultures of *C. perfringens* was also evaluated by Western blot analysis. No extracellular CPE was detected even in 250-fold concentrated supernatants from 8-h sporulating cultures of NCTC 8239 (data not shown). This result, obtained with sporulating cells nearing lysis, confirms that CPE is not secreted from sporulating *C. perfringens* cells prior to lysis of the mother cell. Since all CPE in 8-h sporulating cultures of *C. perfringens* remains intracellular, cell lysates were used to estimate the total amount of CPE produced in sporulating *C. perfringens* cultures. The amount of CPE produced by sporulating cultures of CPE-positive strain NCTC 8239 was determined by using purified CPE standards (50 ng to 1 μg of CPE per lane) dissolved in 10 μg of cell lysates from sporulating cultures of ATCC 3624 (enterotoxin negative; see below). Densitometric scans of six autoradiographs showed that CPE represented approximately 13% of total cell lysate protein produced by sporulating cultures of NCTC 8239. This value agrees well with a previous estimate, obtained by immunoprecipitation (36), of CPE production by sporulating cultures of NCTC 8239. These results indicate that approximately 65 μg of CPE is produced per ml of sporulating NCTC 8239 culture under these sporulation conditions.

When similar conditions for Western analysis were applied to vegetative cultures of NCTC 8239, no CPE was detected in either cell lysates (Fig. 2) or concentrated culture supernatants (data not shown). However, trace amounts of a 35-kDa immunoreactive species were detectable in cell lysates from vegetative cultures of NCTC 8239 if the autoradiograph exposure time was 4 days rather than the normal overnight (data not shown). This trace immunoreactivity was not due to contamination from carryover from other CPE-containing lanes, since trace amounts of this species were also visible on gels loaded only with cell lysate protein from vegetative cultures of NCTC 8239. This trace immunoreactivity does not appear to be due to low-level sporulation in the vegetative culture since (i) phase-contrast microscopic examination did not show the presence of any refractile spores in 10 fields containing 1,000 total bacteria and (ii) there were no heat-resistant spores detected in these vegetative cultures, as detected by plating of heat-shocked culture samples. For comparison, a similar analysis by phase microscopy indicated about 80 to 90% sporulation in Duncan-Strong cultures of NCTC 8239, and plate counts of heat-shocked sporulating NCTC 8239 showed $>10^5$ heat-resistant spores per ml of culture. Quantitation of the amount of CPE in vegetative NCTC 8239 lysates is difficult since the amount was less than the lowest reliably detected CPE standard (50 ng). Therefore, CPE represents less than 0.05% of total cell lysate protein in NCTC 8239 vegetative cultures, i.e., there is less than 50 ng of CPE per ml of vegetative culture of NCTC 8239.

Figure 2 also confirms that ATCC 3624 is CPE negative (as generally accepted; see Discussion) since neither vegetative nor sporulating cultures of this strain produced CPE. Even autoradiographs of ATCC 3624 cell lysates or culture supernatants exposed for 4 days remained negative. The failure to detect CPE in ATCC 3624 was not due to failure to induce sporulation, since phase-contrast microscopic analysis of these cultures showed 60 to 80% sporulation.

**Determination of the relative cytotoxic activity of recombinant CPE.** It was also of interest to determine whether the recombinant CPE made by *E. coli* retains full toxicity. This question is of critical importance for future studies of the CPE structure versus function relationship using CPE fragments and other derivatives produced in an *E. coli* host background. Given that CPE activity can be highly affected by specific proteases which produce very small modifications in CPE size (9, 13) which may be missed by common SDS-PAGE analyses of CPE size and that background proteases (or other protein-modifying enzymes) might differ considerably between *E. coli* and sporulating *C. perfringens*, it was conceivable that CPE activity might be affected by expression in an *E. coli* host background.

To address this question, the cytotoxicity of recombinant CPE was evaluated by using a standard $^{86}$Rb-release Vero cell assay for CPE activity (12). Important control results shown in Fig. 4 indicate that cell lysates from sporulating or vegetative ATCC 3624 cultures or negative control *E. coli* C1

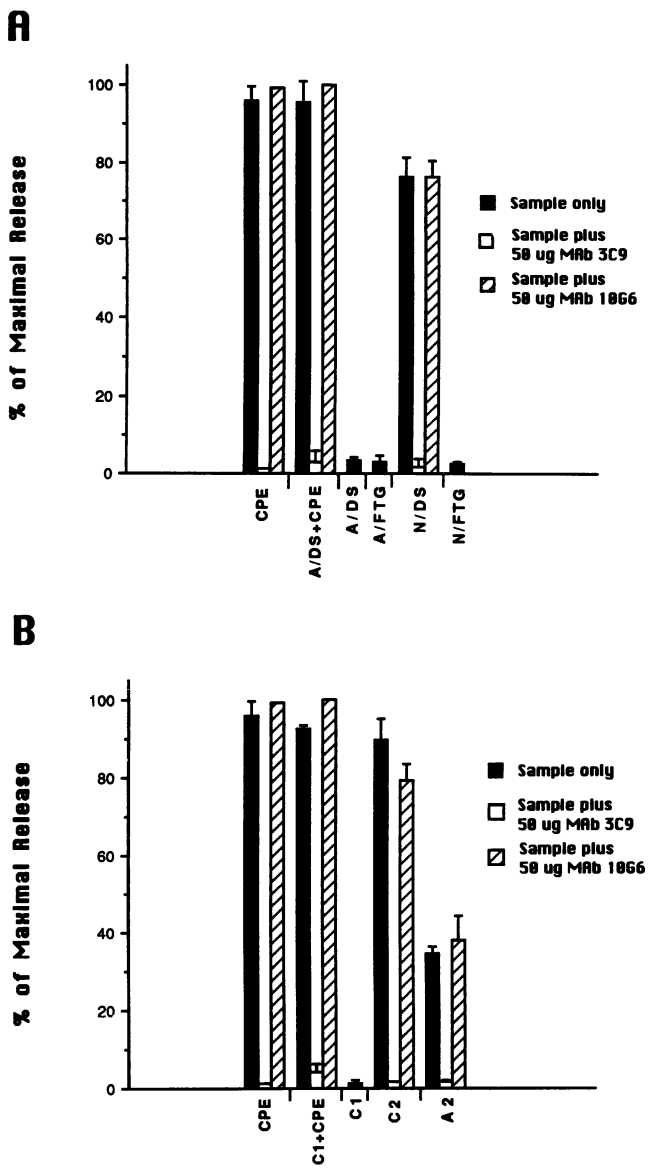


FIG. 4. Cytotoxic effects of *C. perfringens* (A) and *E. coli* (B) lysates. Lysates (see below) or purified CPE (1.25 μg) was preincubated for 15 min in the absence or presence of the CPE-neutralizing MAb 3C9 or non-CPE-neutralizing MAb 10G6. (A) *C. perfringens* lysates included CPE-positive strain NCTC 8239 (abbreviated as N) grown under sporulating conditions (in Duncan-Strong medium [DS], 10 μg of lysate per sample) or vegetative conditions (in fluid thioglycolate [FTG], 2 mg per sample) and CPE-negative strain ATCC 3624 (abbreviated as A) grown under sporulating and vegetative conditions (DS and FTG, respectively; 2 mg per sample for both growth conditions). (B) *E. coli* lysates (1 mg of protein per sample) included lysates from CPE-positive clones A2 and C2 and CPE-negative clone C1. In indicated samples, purified CPE was preincubated with 2 mg of FTG- or DS-grown (not shown) cell lysate from *C. perfringens* ATCC 3624 or 1 mg of cell lysate from *E. coli* clone C1. After preincubation, samples were added to $^{86}$Rb-labeled Vero cells to measure CPE cytotoxicity, as described in Materials and Methods. Results shown are from experiments performed in triplicate with duplicate samples in each experiment. Bars represent the means, while error bars represent the standard errors of the means. Points without error bars had errors too small to depict.

cultures are not cytotoxic. Further, neither control *C. perfringens* nor *E. coli* lysates interfere with CPE cytotoxicity in this assay since full $^{86}$Rb release occurred after 1.25 μg of purified CPE was preincubated with either C1 lysates (Fig. 4B), sporulating ATCC 3624 lysates (Fig. 4A), or vegetative ATCC 3624 lysates (not shown). This conclusion was also supported by experiments demonstrating that equivalent $^{86}$Rb release was produced by standard amounts (0.15 to 2.5 μg of CPE per sample) of purified CPE dissolved in 2 ml of either HBSS alone or HBSS containing either 2 mg of vegetative or sporulating ATCC 3624 lysates or 1 mg of C1 lysate (minimal assay sensitivity was 0.31 μg of CPE per sample; data not shown).

Lysates from both *cpe*-positive *E. coli* clones caused significant sample-induced $^{86}$Rb release (Fig. 4B). This effect appears to result directly from recombinant CPE in these lysates since a neutralizing MAb prepared previously against native CPE produced by *C. perfringens* (44) abolished all sample-induced $^{86}$Rb release caused by both lysates. A control non-neutralizing CPE MAb had no effect on lysate-induced $^{86}$Rb release. Consistent with the Western blot results, strain C2 always had about twofold more cytotoxic activity than strain A2, and overnight cultures for either clone had approximately twice the cytotoxic effects of log-phase cultures. By comparing the activity of A2 and C2 lysates with a cytotoxicity standard curve derived from purified *C. perfringens* CPE dissolved in 2 mg of C1 lysate, it was determined that recombinant CPE represents about 0.1 and 0.2% of total protein in log-phase and overnight C2 cultures, respectively (data not shown). Similarly, by cytotoxic assay, recombinant CPE represents about 0.05 and 0.1% of total protein in log-phase and overnight A2 cultures, respectively. If these values determined by cytotoxic activity are compared with the total amount of CPE protein in *E. coli* lysates estimated by Western blots, they indicate that, on a molar basis, recombinant CPE produced in *E. coli* is fully as active as native enterotoxin made in *C. perfringens*.

Positive-control cytotoxicity studies also confirmed that sporulating cultures of NCTC 8239 produce high amounts of CPE (Fig. 4A). All cytotoxicity associated with this lysate appears to be due to CPE since MAb 3C9 specifically blocks all lysate-induced $^{86}$Rb release. When the amount of cytotoxic CPE activity in lysates from sporulating NCTC 8239 cultures was compared with a cytotoxicity standard curve derived by using purified CPE dissolved in 10 μg of ATCC 3624 sporulating cell lysate, it was determined that CPE activity represented about 10% of total protein. This result is in close agreement with estimates of CPE levels in sporulating lysates which were obtained by Western immunoblots. Results shown in Fig. 4A also indicate that there was no CPE activity detectable in vegetative NCTC 8239 cell lysates. This result indicates that there is less CPE present than the sensitivity limit of this assay, which is 0.31 μg of CPE per 2 mg of cell lysate (i.e., CPE represents less than 0.02% of total lysate protein in these vegetative cultures), confirming our Western analysis estimates which indicated that the amount of CPE produced by vegetative cultures of *C. perfringens* is exceedingly small.

## DISCUSSION

Numerous attempts yielding only partial *cpe* clones have led to the recent proposal by Granum and Stewart that possession of the intact *cpe* gene is highly unfavorable for *E. coli* (9). However, two clones which carry the intact *cpe* gene and express moderate amounts of CPE have now been

identified and characterized in this report. Since nearly 1% of the recombinant clones generated by our cloning protocol were able to strongly hybridize with our *cpe* probe, successful cloning of an intact *cpe* gene by the strategy described in this report does not appear to be an unfavorable event in *E. coli*.

This success now raises the question of why previous attempts to clone *cpe* have been so unsuccessful. The earlier failure of our laboratory to obtain a complete *cpe* clone by using the λgt11 expression vector system coupled with anti-CPE screening is understandable since (i) this technique often identifies fusion proteins from partial clones and (ii) only a single phage producing a protein cross-reactive with anti-CPE was isolated in that study (14). It would also be unlikely that the cloning strategy of Van Damme-Jongsten et al. (41) would yield a complete *cpe* clone since they selected for *Hin*dIII or *Rsa*I restriction fragments of only 0.8 to 1.2 kb, while the *cpe* ORF alone is approximately 1 kb. In their original report (17), Iwanejko et al. selected far larger DNA fragments (6 to 10 kb) than Van Damme-Jongsten et al. did, but they used partial *Hin*dIII digestion to isolate these fragments. Their use of *Hin*dIII for digestion in these attempts significantly lessened the probability of obtaining a complete *cpe* clone since there are two *Hin*dIII sites within *cpe* and another *Hin*dIII site lies immediately upstream of the *cpe* ORF. This contention is supported by the observation that the original *cpe* clone of Iwanejko et al. (17) terminates at nucleotide 873 of the *cpe* ORF, a location which correlates with a *Hin*dIII site (12). The later unsuccessful *cpe* cloning attempts by Iwanejko et al. discussed in the review by Granum and Stewart (9) used either *Eco*RI digestion to generate a 10-kb *cpe*-containing fragment or *Pst*I-*Eco*RI digestion to clone *cpe* as two specific restriction fragments. These appear to be logical approaches which should have produced complete *cpe* clones. It is not possible to speculate on why these unpublished attempts were unsuccessful without knowing how many recombinant colonies were screened or other pertinent information.

As specified in Results and Fig. 1, the A2 *cpe* sequence data have similarities and differences with the *cpe* sequence of Van Damme-Jongsten et al. (41), which was also generated from *C. perfringens* NCTC 8239 source DNA. Among the most notable differences is that the A2 sequence predicts a 319-amino-acid sequence for CPE rather than the 320 amino acids indicated by the sequence of Van Damme-Jongsten et al. This difference results from sequence variation in the extreme 3′ region of *cpe*. Several lines of evidence support the accuracy of the extreme 3′ *cpe* sequence reported for clone A2. First, nucleotide sequences of both clone C2 and a previously reported and independently obtained partial *cpe* clone match exactly the A2 sequence (12). Second, the deduced amino acid sequence for the extreme C terminus of CPE encoded by clone A2 is identical to the amino acid sequence of this region which was generated by amino acid sequencing of purified CPE from NCTC 8239 (8). Finally, the deduced amino acid sequence reported for clone A2 retains CPE-like function. The extreme C terminus of CPE contains most, if not all, of the receptor binding activity of the enterotoxin molecule (12). Earlier studies (12) have shown that a 30-amino-acid synthetic peptide corresponding to the extreme C-terminal CPE sequence encoded by clone A2 retains full CPE-like binding activity. These results do not necessarily imply that the sequence results for the 3′ *cpe* region of Van Damme-Jongsten et al. are incorrect (further discussion below), but additional studies using synthetic peptides which correspond to the 3′ *cpe* sequence of Van Damme-Jongsten et al. or generation of additional independently derived sequence information for the extreme 3′ region of the *cpe* ORF in strain NCTC 8239 would help resolve the identity of this critical region in the enterotoxin molecule. The C terminus of CPE is of current interest not only for its functional activity, i.e., receptor binding, but also because this region contains a neutralizing epitope and is a potential candidate for vaccine development (31); therefore, it is important to establish the proper amino acid sequence of the C terminal region of CPE.

There were a number of other sequence differences for *cpe* from NCTC 8239 noted between the A2 sequence and the sequence of Van Damme-Jongsten et al. There are at least four possible explanations accounting for these differences, including (i) possible divergence of the *cpe* gene of NCTC 8239 between stocks of strain NCTC 8239 held by different laboratories, (ii) strain mislabeling, (iii) mix-up of sequencing results from different *C. perfringens* strains, or (iv) simple sequencing errors. The greatest concentration of sequence differences between the A2 sequence and the sequence of Van Damme-Jongsten et al. occurs in the 3′ half of the *cpe* ORF. As stated above, the A2 and C2 *cpe* ORF sequences agree exactly with each other and with the 3′ sequence generated from a completely independent partial 3′ *cpe* clone (12, 14), making it unlikely that the A2 sequence shown in Fig. 1 contains simple sequencing errors. Granum and Stewart (9) have noted that our previously reported 3′ *cpe* sequence from NCTC 8239 (which matches exactly the A2 sequence) more closely resembles the *cpe* ORF sequence results obtained by Van Damme-Jongsten et al. for *C. perfringens* F3686 than it does their results for strain NCTC 8239. This might suggest the possibility of either strain mislabeling or a mix-up of sequencing or cloning results between strains as an explanation for differences between studies. This possibility cannot be totally excluded, but such mishaps are unlikely to have occurred on our part since our laboratory does not have strain F3686 and, unlike Van Damme-Jongsten et al., we worked with only one *C. perfringens* strain (i.e., NCTC 8239) as a source of DNA. This leaves open the possibility of divergence of the *cpe* gene between different stocks of the same strain. This seems the most likely explanation of the sequencing differences reported between separate labs if the partial *cpe* sequence from NCTC 8239 of Iwanejko et al. (9) is also included in the overall comparison of *cpe* ORF sequences from strain NCTC 8239. Of the six sequencing differences noted between the A2 sequence and the sequence of Van Damme-Jongsten et al. which are also covered by the partial clone of Iwanejko et al., the sequence of Iwanejko et al. (9) matches the A2 sequence at three differences and the sequence of Van Damme-Jongsten et al. at three differences. Unfortunately, the sequence of Iwanejko et al. does not include the extreme 3′ region of the *cpe* ORF or adjacent downstream regions which together contain the greatest variability between the A2 sequence and the sequence of Van Damme-Jongsten et al.

If these independent sequencing results are suggesting that some microheterogeneity occurs in the *cpe* ORF in different stocks of the same *C. perfringens* strain, it will be interesting to compare more extensively *cpe* ORF sequences generated from other enterotoxigenic *C. perfringens* food poisoning isolates. It will be also be interesting to compare *cpe* ORF sequences from *C. perfringens* isolates obtained from nonfood-borne, CPE-linked human diseases or from nonhuman animal hosts to better identify conserved and variable re-

gions of the CPE protein. A concise determination of the extent of microheterogeneity in *cpe* sequences will clearly require sequence comparisons from more strains than the two that are currently available.

A final comment on sequence differences between our results and those of Van Damme-Jongsten et al. focuses on the region downstream from the *cpe* ORF. Our sequence generates a putative stem-loop structure followed by an oligo-T tract, i.e., a region typical of a rho-independent transcriptional terminator (32, 46). Van Damme-Jongsten et al. did not identify this putative structure in their report. Their sequence does not generate as strong an inverted repeat in this downstream region, and they did not extend their 3′ sequence into the oligo-T tract detected in the A2 sequence.

As mentioned in Results, the A2 *cpe* sequencing data indicate that the *cpe* ORF encodes a protein of 319 amino acids. Since (i) mature CPE also contains approximately 319 amino acids (8) which match the ORF-predicted sequence of *cpe* in clone A2, (ii) there are in-frame termination codons immediately preceding and following this putative *cpe* ORF, and (iii) recombinant CPE made in *E. coli* comigrates with native mature CPE, these results argue strongly against an early proposal by Smith and McDonel (36) that CPE is initially synthesized as a 52-kDa precursor in *C. perfringens*. It is likely that the 52-kDa "precursor" band they observed on SDS-PAGE following immunoprecipitation is due to anomalous aggregation of 35-kDa CPE (27). Similar CPE aggregate bands of 50 to 52 kDa have been observed when native CPE was analyzed by SDS-PAGE (28). The current evidence against CPE being processed from a larger precursor does not, of course, eliminate other proposals that 35-kDa CPE itself may be subsequently cleaved in the intestine by intestinal proteases (see references 23 and 24 for reviews).

Our results suggest that CPE expression in *E. coli* is largely driven from a clostridial promoter. There have been previous reports suggesting that other *C. perfringens* toxins can also be synthesized in *E. coli* from clostridial promoters. Expression of these other *C. perfringens* toxins in *E. coli* has generally been higher than our CPE results. For example, previous reports suggest that *E. coli* synthesizes about 10 μg of *C. perfringens* alpha-toxin per ml and 5 to 25 μg of *C. perfringens* epsilon-toxin per ml (16, 32, 40). At least some of these differences in expression levels might result from the fact that epsilon- and alpha-toxins, unlike CPE, are not sporulation-linked toxins. It may be easier for *E. coli* to recognize vegetative clostridial promoters than the *cpe* promoter, but analysis of this question awaits further study using different *C. perfringens* toxin genes cloned into similar vectors and background host strains.

The quantitative evaluation of CPE expression between recombinant *E. coli* and vegetative or sporulating *C. perfringens* cultures suggests several interesting conclusions. First, these results suggest that sporulation is not essential for CPE synthesis but does strongly facilitate expression of the *cpe* gene. This hypothesis is supported by the data indicating that (i) *E. coli* (a nonsporulating organism) produces moderate amounts of CPE, apparently from a clostridial promoter, (ii) vegetative *C. perfringens* cultures can produce trace amounts of CPE, and (iii) a sporulating culture of *C. perfringens* NCTC 8239 can express amounts of CPE several orders of magnitude larger than either recombinant *E. coli* (even though the recombinant *E. coli* clones contain more than one copy of *cpe* per cell, while *C. perfringens* NCTC 8239 has only a single copy of *cpe* [6]) or vegetative cultures of the same *C. perfringens* strain. Second, this study describes the first definitive proof of CPE expression by vegetative *C. perfringens* cultures. At least two early studies (7, 10) purported to demonstrate low-level CPE expression by vegetative cultures of *C. perfringens* by using serologic techniques. However, unlike the current study, neither of these previous studies specifically demonstrated the presence of a 35-kDa species in vegetative *C. perfringens* cultures. This is important since the very small amounts of vegetative "CPE" detected in these previous enzyme-linked immunosorbent assays or immunoprecipitation studies could have been due to serum reactivity with other antigens such as spore coat proteins (see references 28 and 32 for review of possible antigenic cross-reactivities between CPE and spore coat proteins). This criticism seems important and valid since one of these previous studies (7) reported detection of CPE in ATCC 3624 vegetative lysates, a strain which has usually been considered CPE negative (25). In the current study, no CPE was detected in either vegetative or sporulating ATCC 3624 cell lysates by Western blots, and DNA from this strain did not hybridize with either the 0.5-kb *cpe* probe or the 5.7-kb insert containing the intact *cpe* gene (data not shown). This strongly suggests that the previous immunoprecipitation results indicating CPE expression by ATCC 3624 which were gathered by immunoprecipitation techniques may have been artifactual.

A final interesting feature of CPE expression to compare between *E. coli* and *C. perfringens* concerns the cellular location of CPE after synthesis. In agreement with previous studies reporting that CPE accumulates intracellularly in sporulating *C. perfringens* and is released only upon lysis of the mother cell to release the free endospore (see references 23, 24, and 28 for reviews), the Western blot data generated in this study clearly indicate an intracellular location for CPE in the sporulating *C. perfringens* cell, i.e., there was no secreted CPE detected in *C. perfringens* culture supernatants even after 250-fold concentration. However, a considerable portion of the CPE produced in *E. coli* was found to localize in the periplasm, consistent with CPE secretion through the cytoplasmic membrane of this organism. Periplasmic localization of CPE occurred in *E. coli* even though *cpe* does not encode a typical N-terminal signal sequence. It remains possible that the cell export machinery in *E. coli* is recognizing some other feature of the CPE protein since *E. coli* is known to have other periplasmic secretion mechanisms besides use of N-terminal signal sequences (21). For example, *E. coli* hemolysin export sequences appear to be localized at the C terminus (21). However, comparison of the C-terminal region of CPE with *E. coli* hemolysin did not indicate obvious similarities, so the mechanism accounting for CPE accumulation in the periplasm of *E. coli* remains unknown.

Because of the extreme AT content of *C. perfringens* DNA, we have followed warnings (32, 46) not to identify putative *cpe* promoter sequences by visual examination. Experimental analysis to determine the actual *cpe* promoter sequences is currently under way. When these results are available, it will be interesting to compare *cpe* promoter sequences with (i) promoters for vegetatively expressed *C. perfringens* toxins and (ii) sporulation-associated promoters of *Bacillus* and *Clostridium* spp. Studies to compare transcriptional and translational control of CPE synthesis in sporulating *C. perfringens* cells are also ongoing. It will be interesting to determine whether the putative stem-loop structure identified downstream of *cpe* is involved in transcriptional termination or message stability. An early study

(18) suggests that the enterotoxin message may be extraordinarily stable, but this should be reinvestigated by modern techniques.

Clearly, the molecular genetics behind the abundant synthesis of CPE by sporulating *C. perfringens* remains an intriguing topic in gene regulation. Recent studies suggest that *cpe* may be part of a mobile genetic element integrated into the *C. perfringens* chromosome (3). The availability of cloned copies of *cpe* should be invaluable for investigating the distribution of *cpe* and the regulation of CPE expression.

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