INFECTION AND IMMUNITY, Aug. 1996, p. 3301–3309 Vol. 64, No. 8
0019-9567/96/$04.00+0

Regulated Expression of *Clostridium perfringens* Enterotoxin in Naturally *cpe*-Negative Type A, B, and C Isolates of *C. perfringens*

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Received 28 December 1995/Returned for modification 18 March 1996/Accepted 6 May 1996

***Clostridium perfringens* enterotoxin (CPE), the virulence factor responsible for symptoms associated with *C. perfringens* type A food poisoning, is produced by enterotoxigenic *C. perfringens* type A isolates when these bacteria sporulate in the gastrointestinal tract. Less than 5% of the global *C. perfringens* population apparently carries the *cpe* gene. To assess the distribution of *cpe*-regulatory factors, we investigated whether the *cpe* gene of a *C. perfringens* food poisoning isolate can be expressed and properly regulated (i.e., expressed in a sporulation-associated manner) when transformed into naturally *cpe*-negative *C. perfringens* isolates. Sporulation-associated CPE expression was observed when low-copy-number plasmids carrying either a 5.7-kb DNA insert, containing the *cpe* open reading frame plus >1 kb each of upstream and downstream flanking sequences from *C. perfringens* food poisoning isolate NCTC 8239, or a 1.6-kb insert, containing only the *cpe* open reading frame of NCTC 8239, were electroporated into *cpe*-negative *C. perfringens* type A, B, and C isolates. Northern (RNA) blot analysis demonstrated that the sizes of the *cpe* message in the transformants and the naturally enterotoxigenic *C. perfringens* NCTC 8239 were similar and that this message was detectable only in sporulating cultures of the transformants or NCTC 8239. These studies strongly suggest that many, if not all, *cpe*-negative *C. perfringens* isolates (including type B isolates, which are not known to naturally express CPE) produce a factor(s) involved in normal (i.e., sporulation-associated) transcriptional regulation of CPE expression by *C. perfringens* food poisoning isolates. These findings are consistent with this CPE-regulatory factor(s) also regulating the expression of other genes in *C. perfringens*.**

Clostridium perfringens is an endospore-forming, gram-positive bacterium that ranks among the most important of the anaerobic pathogens affecting humans and domestic animals (17, 20). The virulence of *C. perfringens* derives largely from its prolific ability to express protein toxins (17, 20). However, each individual *C. perfringens* cell produces only a limited subset of the total repertoire of known *C. perfringens* toxins (17, 20). Consequently, a typing scheme has been developed for classifying *C. perfringens* into five types (A to E), on the basis of each isolate's pattern of expression of 4 (alpha, beta, epsilon, and iota) of the 13 known *C. perfringens* toxins (17, 20). Each of the five types of *C. perfringens* has been associated with specific human or veterinary diseases (17, 20).

One of the most common human diseases caused by *C. perfringens* is *C. perfringens* type A food poisoning, which annually ranks among the leading foodborne diseases in industrialized countries (4). The symptoms (diarrhea and cramping) of this food poisoning are caused by *C. perfringens* enterotoxin (CPE), a single 35-kDa polypeptide with a unique amino acid sequence and mechanism of action (9, 16, 29). Recent molecular epidemiology surveys (14, 28) suggest that only a low percentage (~5%) of all *C. perfringens* isolates carry the *cpe* gene. While nearly all cases of *C. perfringens* type A food poisoning involve enterotoxigenic type A *C. perfringens* isolates, expression of CPE by some type C and D *C. perfringens* isolates has also been described (24).

At least two features make CPE expression an interesting problem in prokaryotic gene regulation. First, the expression of CPE by *C. perfringens* food poisoning isolates is highly regulated, with sporulating cultures of *C. perfringens* NCTC 8239 producing at least 1,500-fold more CPE than vegetative cultures of this strain (9). Second, this sporulation-associated CPE expression often reaches extremely high levels, with CPE representing 10% or more of the total protein in sporulating cells of some *C. perfringens* food poisoning isolates (9). Since CPE is not an actively secreted protein but instead is released when the mother cell lyses at the completion of sporulation, so much intracellular CPE accumulates during sporulation that CPE-containing paracrystalline inclusion bodies form in the cytoplasm of many sporulating *C. perfringens* cells (15).

Currently, the molecular basis for regulation of CPE expression is not well understood but is being actively studied (5–7, 9, 18). Recent studies suggest that high-level expression of CPE by some *C. perfringens* food poisoning isolates is not explainable by *cpe* gene amplification, since these isolates appear to contain only a single chromosomal copy of the *cpe* gene (5–7). Another recent study (18) has identified possible *cpe* promoter regions and also presented some RNA slot blot and reporter construct data supporting transcriptional regulation of CPE expression in food poisoning isolates.

The abundant, yet highly regulated, expression of CPE from the single chromosomal *cpe* gene present in food poisoning isolates strongly suggests that growth phase-specific *cpe*-regulatory factors may be actively involved in CPE expression. One of many interesting questions about CPE expression that has not been addressed to date concerns the distribution of *cpe*-

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristics	Source or reference
C. perfringens		
NCTC 8239	Type A, *cpe* positive	14
ATCC 3624	Type A, *cpe* negative	14
PS49	Type B, *cpe* negative	Ronald Labbe
CN 5383	Type C, *cpe* negative	14
E. coli DH5α		9
Plasmids		
pJIR418	*C. perfringens-E. coli* shuttle plasmid; Cm^r Em^r	25
pJRC100	5.7-kb *Xba*I *C. perfringens* DNA fragment, containing the *cpe* ORF, ligated into pJIR418	This work
pJRC200	1.6-kb PCR fragment, containing the *cpe* ORF, inserted into pJIR418	This work
pC1	5.7-kb *Xba*I *C. perfringens* DNA fragment, not containing the *cpe* ORF, ligated into pUC19	9
pA2	5.7-kb *Xba*I *C. perfringens* DNA fragment, containing the *cpe* ORF, ligated into pUC19; isolated from *E. coli* DH5α transformant A2	9
pC2	Same as pA2 except isolated from *E. coli* DH5α transformant C2	9

regulatory factors among *C. perfringens* isolates; i.e., do all *C. perfringens* isolates produce *cpe*-regulatory factors, or are these factors made only by the enterotoxigenic isolates that apparently represent <5% of the total *C. perfringens* population? To begin to address this question, we asked what would happen if the *cpe* gene from *C. perfringens* food poisoning isolate NCTC 8239 was introduced, on a low-copy-number shuttle plasmid, into naturally *cpe*-negative *C. perfringens* type A, B, and C strains. The results from these studies strongly suggest that naturally *cpe*-negative isolates of *C. perfringens* produce some or all of the regulatory factors involved in CPE expression by food poisoning isolates, since all three naturally *cpe*-negative isolates were able to express large amounts of CPE in a strictly sporulation-associated manner after being transformed with the *cpe* gene from NCTC 8239.

MATERIALS AND METHODS

Bacteria and growth conditions. All bacteria and plasmids used in these studies are described in Table 1. *Escherichia coli* DH5α cultures were grown overnight, with shaking, at 37°C in M9 medium (9). Vegetative *C. perfringens* cultures were grown for 9 h at 37°C in fluid thioglycolate broth (FTG) (Difco). Unless otherwise noted, a modified Duncan-Strong (MDS) medium, which was prepared similarly to Duncan-Strong sporulation medium (14) except that peptone (Difco) was substituted for Proteose Peptone and the medium was supplemented with 200 μg of theophylline (Sigma) per ml, was used to obtain sporulating cultures of *C. perfringens*. The modifications for MDS medium were used because previous observations indicated that some *C. perfringens* isolates sporulate better when grown in the presence of theophylline or peptone (8, 21). MDS cultures of *C. perfringens* were grown for 8 h at 37°C. All media were supplemented with 15 to 20 μg of chloramphenicol per ml or 100 μg of ampicillin per ml for growing transformants containing pJIR418-based or pUC19-based plasmid constructs, respectively.

Cell protein levels in *C. perfringens* cultures were determined with washed cells that were sonicated and then assayed by using the Bio-Rad protein assay with bovine serum albumin as the protein standard.

Plasmid construction. Standard recombinant DNA techniques and reagents (23) were used in DNA manipulations involved in the construction of pJRC100 and pJRC200.

Briefly, the 5.7-kb *Xba*I fragment of *C. perfringens* NCTC 8239 DNA containing the complete *cpe* open reading frame (ORF) was excised from the recombinant plasmid pC2 (Table 1) and then ligated into the *C. perfringens-E. coli* shuttle plasmid pJIR418 (Table 1), creating a 13.1-kb plasmid named pJRC100.

To construct pJRC200, two primers, 5′-GCTCTAGACTTGTTGGGTGATT TATTACTCC-3′ and 5′-CCGGAATTCTATATGGAAGGAGAAATTAATG C-3′, were designed. These primers were used in a standard PCR amplification (23), with the *Xba*I insert of pJRC100 as the template, and the resultant 1.6-kb PCR product was ligated into pJIR418 to create a 9-kb plasmid named pJRC200. Nucleotide sequencing of the entire insert of pJRC200 (data not shown) confirmed that (i) the 1.6-kb PCR product contains the intact *cpe* ORF plus ~0.4 kb of upstream and ~0.2 kb of downstream sequence (which may contain *cis* regulatory elements [5, 9, 18]), (ii) this 1.6-kb sequence does not include, in any reading frame, any other ORFs encoding polypeptides of >~30 amino acids, and (iii) no PCR-induced sequence errors were introduced during amplification.

Transformation of *C. perfringens* and *E. coli* with pJRC100 and pJRC200. *C. perfringens* cells were transformed by using a modification of a previously described electroporation method (19). Cells were grown at 37°C to late log phase or early stationary phase in TGY broth (3% Trypticase, 2% glucose, 1% yeast extract, and 0.1% cysteine), washed twice with SMP buffer (270 mM sucrose, 1 mM $MgCl_2$, and 7 mM $NaPO_4$, pH 7.3), and then resuspended in 1 ml of SMP buffer to produce a 25-fold concentration of these cells. The washed cells (200 μl) plus plasmid DNA (5 μg) were added to cold 0.4-cm cuvettes (Bio-Rad). After a 1-min incubation on ice, the cuvette containing the cell-plasmid DNA mixture was placed in a Bio-Rad Gene Pulser set for 2.5 kV and 25 μF, with the pulse controller set to 200 Ω. Immediately after the pulsing, the cell suspension was incubated on ice for 10 min, and then 3 ml of warm (37°C) TGY broth was added and the mixture was incubated for 3 h at 37°C to facilitate recovery of the transformants. Aliquots (100 to 1,000 μl) of this mixture were then plated on brain heart infusion agar (Difco) containing chloramphenicol (15 to 30 μg/ml). These plates were incubated for 24 h at 37°C in an anaerobic atmosphere, created by using a GasPak jar, in order to allow growth of chloramphenicol-resistant transformants.

Classical $CaCl_2$-mediated transformation (23) of early- to mid-log-phase cultures was used to introduce pJRC100 and pJRC200 into *E. coli* DH5α.

DNA isolation for Southern blots. A previously described protocol (14) was used, with modification, to isolate total *C. perfringens* DNA for Southern blotting experiments. Overnight cultures of *C. perfringens* grown in 10 ml of TGY broth (supplemented with 15 μg of chloramphenicol per ml for growth of transformants) were washed twice with 1× TES (0.2 M Tris, 0.1 M EDTA, and 0.2 M NaCl). Cell pellets were treated with lysozyme solution (14) and incubated for 2 h at 37°C. Protoplasts were pelleted by microcentrifugation and incubated in 600 μl of 100 mM Tris–10 mM EDTA containing 2.5% Sarkosyl (Sigma) for 10 min at 60°C to obtain lysis. DNA was then purified from the lysates by organic extraction as described previously (23).

Total *E. coli* DNA was obtained from a 10-ml Luria broth (supplemented with either 15 μg of chloramphenicol per ml or 100 μg of ampillicin per ml for growing transformants) culture grown overnight at 37°C, with aeration, by the method described above for obtaining total *C. perfringens* DNA. *E. coli* plasmid DNA was obtained by a standard small-scale alkaline lysis method (23).

Preparation of a DIG-labeled, *cpe*-specific probe. A 639-bp digoxigenin (DIG)-labeled, double-stranded, *cpe*-specific DNA gene probe was prepared by a modification of a standard two-step PCR amplification method (10). Briefly, nucleotides 205 to 843 of the *cpe* ORF were first PCR amplified from the *cpe*-containing plasmid pA2 (Table 1) by using the following primers 5′-GGTACC TTTAGCCAATCA-3′ and 5′-TCCATCACCTAAGGACTG-3′. This PCR utilized 500 ng of template, 1 μM each primer, 1 mM deoxynucleoside triphosphates (Perkin-Elmer), 2.5 mM $MgCl_2$, and 2.5 U of *Taq* polymerase (Boehringer-Mannheim). The reaction mixture, in a total volume of 50 μl, was placed in a thermal cycler (Perkin-Elmer) for 30 cycles, each consisting of 2 min at 94°C, 2 min at 55°C, and 2 min at 72°C.

The 639-bp PCR product obtained from this amplification was then used as the template for a second PCR in order to produce a 639-bp PCR product containing DIG-labeled nucleotides. This second PCR involved the incubation of 50 ng of template (i.e., the 639-bp product amplified in the previous PCR); 2.5 U of *Taq* polymerase; 2.5 mM $MgCl_2$; 100 μM dATP, dCTP, and dGTP; 65 μM dTTP; and 35 μM DIG-dUTP (Boehringer-Mannheim) under the same amplification conditions used for the initial PCR described above.

Southern blots. DNA samples were digested to completion with *Eco*RI and/or *Xba*I, separated by electrophoresis on 0.8% agarose gels, transferred to positively charged nylon membranes (Nytran Maximum-Strength Plus; Schleicher and Schuell), and UV fixed to these membranes (23). The 639-bp DIG-labeled *cpe* gene probe, prepared as indicated above, was hybridized to these blots as described by Boehringer Mannheim (4a). The blots were washed at high stringency as described by Sambrook et al. (23). The hybridized probe was detected by using a DIG-chemiluminescence detection system with the Lumi-Phos 530 substrate (Boehringer Mannheim).

Determination of plasmid copy number. The plasmid copy number for each *E. coli* transformant was determined as described previously (27).

Because some *C. perfringens* strains used in these studies also contain natural plasmids, it was necessary to modify the standard method (27) in order to determine the specific pJRC100 and pJRC200 copy numbers in each *C. perfringens* transformant. For this purpose, plasmid fractions were isolated from radiolabeled sporulating and vegetative cultures (grown under standard conditions

except for the addition of 6.5 μCi of [^{3}H]thymidine) of ATCC 3624, CN 5383, or PS49, and from pJRC100 and pJRC200 transformants of each of these strains, by standard methods (23) except that bacterial pellets were resuspended and incubated in a modified solution 1 supplemented with 10 mg of lysozyme per ml for 1 h at 37°C. The resultant plasmid fractions were then digested with *Eco*RI (which cuts only once in pJRC100 and pJRC200), and this *Eco*RI-digested plasmid DNA was run on 0.8% low-melting-point agarose gels. A gel slice corresponding to the size of linearized pJRC100 or pJRC200, as appropriate, was cut from gel lanes of both the transformant and its nontransformed parent strain, and the radioactivity present in each slice was quantitated with a scintillation counter. Radioactivity specifically associated with pJRC100 or pJRC200 was determined by subtracting the counts present in gel slices taken from lanes containing samples obtained from the nontransformed parent strain from the counts present in corresponding gel slices taken from lanes containing transformant samples. These corrected values were then used to calculate (27) pJRC100 and pJRC200 copy numbers for each transformant.

Western blots (immunoblots). Cultures were grown under standard conditions and then sonicated until >95% of all cells were lysed (lysis progress was continuously monitored by phase-contrast microscopy). Following sonication, each culture lysate was analyzed for the presence of CPE by using Western immunoblots (14), and the amount of CPE present in CPE-positive lysates was quantitated by densitometric comparisons against a series of dilutions (50 to 250 ng per lane) of purified CPE, as described previously (9).

Determination of numbers of vegetative and sporulating cells in *C. perfringens* cultures. The numbers of vegetative cells and refractile endospores (i.e., sporulating cells) present per milliliter of sporulating *C. perfringens* culture were determined microscopically with a Hawksley counting chamber as described previously (13). At least 64 squares (enough squares were counted to ensure that at least 600 cells were counted for every sample) of the counting chamber were examined with a phase-contrast microscope (Nikon). Values shown represent means from at least three independent growths of each strain or transformant.

Northern (RNA) blots. RNA was isolated from *C. perfringens* cells by a modification of the protocol supplied with the RNeasy Total RNA Kit (Qiagen). FTG or MDS cultures of *C. perfringens* were harvested by centrifugation, washed, and resuspended in lysis buffer (30 mM Tris, 100 mM NaCl, 5 mM EDTA, and 1% sodium dodecyl sulfate) (3). After 5 min at 37°C, the treated cells were quickly frozen in a dry ice-ethanol bath and then quickly thawed in a 37°C water bath. An equal volume of RNase-inhibiting buffer (RLT lysis buffer [Qiagen]) was added to the cell suspension, and the mixture was subjected to three bursts (10 s each) of sonication to complete cell lysis (3). Each lysate was then homogenized with a Qiashredder column (Qiagen), and total RNA was isolated by application of the resultant homogenate to RNeasy spin columns (Qiagen) followed by elution with DEPC-treated distilled water, as described in the RNeasy kit instructions supplied by Qiagen. The concentration and purity of each RNA preparation were determined by measuring its A_{260} and A_{280} in a spectrophotometer.

Isolated RNA (15 μg) was denatured and run on 1.2% agarose gels containing formaldehyde as described previously (23), transferred to nitrocellulose membranes (Immobilon NC Plus [Millipore]) by capillary elution, and then UV fixed to these membranes. Our DIG-labeled, 639-bp internal *cpe* gene probe was hybridized to the RNA-containing membranes by the procedure described by Boehringer Mannheim (4a). The membranes were then washed under highly stringent conditions (23) with DEPC-treated buffers in RNase-free labware. Finally, the hybridized probe was detected by chemiluminescence with the Lumi-Phos 530 substrate (Boehringer Mannheim).

DNase and RNase treatment of RNA samples. Eight units of RQ1 RNase-free DNase (Promega) was incubated with 15 μg of the RNA sample (prepared as described above) for 30 min at 37°C. The RNA was then immediately ethanol precipitated and subjected to Northern analysis as described above. The ability of the RQ1 RNase-free DNase preparation to digest DNA was confirmed by demonstrating (data not shown) that 0.5 U of the same DNase preparation was able to totally digest 200 ng of linearized pUC19 DNA.

Ten microliters of a 10-mg/ml DNase-free RNase solution (Sigma) was incubated with 15 μg of the RNA sample (prepared as described above) for 30 min at 37°C, and the RNA was then subjected to ethanol precipitation and Northern analysis as described above. The specificity of the RNase preparation was confirmed by demonstrating that no degradation of 200 ng of linearized pUC19 DNA occurred (data not shown) after incubation with a 0.5-μl aliquot of this RNase preparation for 30 min at 37°C.

RESULTS

Southern analysis of *C. perfringens* ATCC 3624, PS49, and CN 5383. Our initial step was to conclusively identify several *C. perfringens* isolates as naturally *cpe* negative. Southern analysis (see Materials and Methods) indicated that DNA from the naturally enterotoxigenic *C. perfringens* food poisoning strain NCTC 8239 hybridized to our *cpe*-specific probe, while DNA from *C. perfringens* ATCC 3624 (a type A strain) or CN 5383 (a type C strain) did not react with this probe (Fig. 1). These

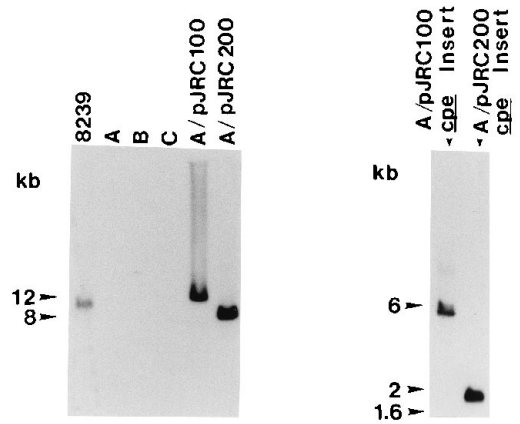

FIG. 1. Southern hybridization analysis of total DNAs from *C. perfringens* strains and transformants. Blots were probed with a 639-bp DIG-labeled *cpe*-specific fragment. (Left) *Eco*RI-digested DNAs from strains NCTC 8239 (8239), ATCC 3624 (A), PS49 (B), and CN 5383 (C) and from the ATCC 3624 transformants containing either pJRC100 (A/pJRC100) or pJRC200 (A/pJRC200). (Right) *Xba*I-digested DNA isolated from the ATCC 3624/pJRC100 transformant (left lane) and *Xba*I-*Eco*RI-digested DNA isolated from the ATCC 3624/pJRC200 transformant (right lane). Molecular sizes of the DNA markers are given to the left of each blot.

results are in agreement with previous classifications of these three strains obtained by using *cpe* PCR assays, *cpe* gene probe dot blot assays, and CPE Western immunoblots (14), confirming that ATCC 3264 and CN 5383 are naturally *cpe* negative and also indicating the reliability of our Southern blot procedure for distinguishing between *cpe*-positive and *cpe*-negative strains of *C. perfringens*. When a similar Southern analysis was applied to DNA samples isolated from *C. perfringens* PS49 (a type B strain), no probe hybridization was detected (Fig. 1).

Identification of PS49, ATCC 3624, and CN 5383 as *cpe*-negative isolates was also independently confirmed by PCR analysis of DNAs isolated from these strains. With a standard *cpe* PCR detection protocol (14), no *cpe* PCR product was amplified from DNA isolated from PS49, ATCC 3624, or CN 5383, although the expected *cpe* PCR product was amplified from five other *C. perfringens* strains, including NCTC 8239, known to be enterotoxigenic (data not shown).

CPE expression by pJRC100 transformants. A 13.1-kb plasmid named pJRC100, composed of a 5.7-kb *Xba*I *cpe*-containing *C. perfringens* DNA insert from strain NCTC 8239 ligated into the *C. perfringens-E. coli* shuttle plasmid pJIR418, was prepared (see Materials and Methods) and then introduced into *cpe*-negative strains ATCC 3624, PS49, and CN 5383 by electroporation. Putative transformants capable of growth on chloramphenicol-containing medium were then selected. Southern blot analysis with our *cpe*-specific probe demonstrated the presence of an ~13-kb band in a chloramphenicol-resistant isolate named ATCC 3624(pJRC100) (Fig. 1), as well as in chloramphenicol-resistant isolates named PS49 (pJRC100) and CN 5383(pJRC100) (data not shown). Southern analysis with the *cpe*-specific probe also demonstrated the specific presence of a 5.7-kb *cpe*-containing fragment in *Xba*I-digested DNA isolated from ATCC 3624(pJRC100) (Fig. 1), as well as in *Xba*I-digested DNAs isolated from PS49(pJRC100) and CN 5383(pJRC100) (data not shown), as would be expected if each of these transformants was stably carrying pJRC100 without any recombinational events having occurred within the 5.7-kb *Xba*I insert ligated into this plasmid. Finally, no *cpe* probe reactivity against ATCC 3624 transformed with the pJIR418 vector alone was detected (data not shown), confirm-

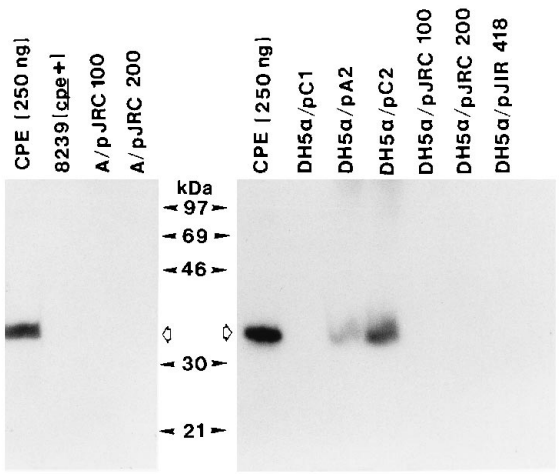

FIG. 2. Western immunoblot analysis of *E. coli* and vegetative *C. perfringens* culture lysates. Culture lysates were analyzed by immunoblot analysis for CPE expression with CPE antiserum and ^{125}I-protein A, as described in Materials and Methods. (Left) Each lane contains 80 μl of vegetative FTG culture lysate from either the naturally enterotoxigenic *C. perfringens* strain NCTC 8239 (8239 [*cpe*$^+$]) or *C. perfringens* ATCC 3624 transformed with either pJRC100 (A/pJRC100) or pJRC200 (A/pJRC200). (Right) Each lane contains 80 μl of culture lysate from *E. coli* DH5α transformed with the plasmid specified (Table 1). For comparison, 250 ng of purified native CPE is shown in the leftmost lane of each blot. The open arrows indicate the migration of purified CPE stained by Coomassie blue (not shown).

ing this probe's specificity for the *cpe*-containing insert of the pJRC100 transformants.

Having obtained evidence for successful stable transformation of ATCC 3624(pJRC100), PS49(pJRC100), and CN 5383 (pJRC100), it was then appropriate to ask whether any of these pJRC100 transformants were able to express CPE and, if so, whether this CPE expression was constitutive or regulated, i.e., whether CPE expression by these transformants would be a sporulation-associated event similar to CPE expression by naturally enterotoxigenic *C. perfringens* food poisoning isolates.

Control experiments confirmed the specificity and reliability of the CPE Western immunoblot assay used to assess CPE expression by the pJRC100 transformants. With this assay, no immunoreactivity against lysates from either sporulating or vegetative cultures of nontransformed ATCC 3624, PS49, or CN 5383 was detected (data not shown), as would be expected since these strains lack the *cpe* gene (Fig. 1). However, a 35-kDa species, which reacts with CPE antiserum and comigrates with purified CPE, was detected in lysates from sporulating, but not vegetative, cultures of NCTC 8239 (Fig. 2 and 3), as would be expected from previous CPE Western immunoblot studies of this strain (9, 14).

When the same CPE Western immunoblot procedure was applied to culture lysates of the pJRC100 transformants, no detectable CPE expression was observed in vegetative cultures of ATCC 3624(pJRC100), PS49(pJRC100), or CN 5383 (pJRC100), even when maximal sample volumes (80 μl) of lysates were analyzed (Fig. 2). However, a 35-kDa immunoreactive band comigrating with purified CPE was detected in lysates of sporulating cultures of all three pJRC100 transformants (Fig. 3). As would be expected if the 35-kDa immunoreactive species present in lysates of these pJRC100 transformants was actually CPE, no 35-kDa species was detected in lysates from sporulating or vegetative cultures of ATCC 3624 transformed with the pJIR418 vector alone (Fig. 2 and 3).

Since there was less than a two- to threefold difference in the amount of cell protein present per milliliter in any culture of these pJRC100 (or pJIR418) transformants (data not shown), the detected differences in CPE expression between sporulating and vegetative cultures are not explainable by differences in growth.

CPE expression by pJRC200 transformants. The results presented in Fig. 2 and 3 clearly establish that all three pJRC100 transformants express CPE in a sporulation-associated pattern that mimics CPE expression by naturally enterotoxigenic *C. perfringens* food poisoning isolates, indicating either that all three of these naturally *cpe*-negative isolates are producing a *cpe*-regulatory factor(s) or that a *cpe*-regulatory factor(s) is encoded by a gene located in an upstream or downstream ORF adjacent to the *cpe* ORF and, thus, this regulatory gene has been carried into the pJRC100 transformants as part of the 5.7-kb *Xba*I insert.

To distinguish between these two possibilities, a 1.6-kb DNA product containing the intact *cpe* ORF (but no other ORFs in any reading frame), along with short stretches of upstream and downstream sequences that may contain *cis* regulatory elements necessary for expression of the *cpe* gene, was PCR amplified and ligated into pJIR418 (see Materials and Methods), creating pJRC200. pJRC200 was introduced into ATCC 3624, PS49, and CN 5383 cells by electroporation, and chloramphenicol-resistant colonies were selected. Southern analysis confirmed the presence of pJRC200 in three chloramphenicol-resistant transformants, which were named ATCC 3624 (pJRC200), PS49(pJRC200), and CN 5383(pJRC200). All three of these transformants were shown (Fig. 1 and data not shown) to carry a 9-kb *cpe*-containing plasmid, which corresponds to the expected size of pJRC200, and a 1.6-kb *Eco*RI-*Xba*I DNA fragment that hybridized our *cpe*-specific probe; i.e., there was no evidence that recombinational events had

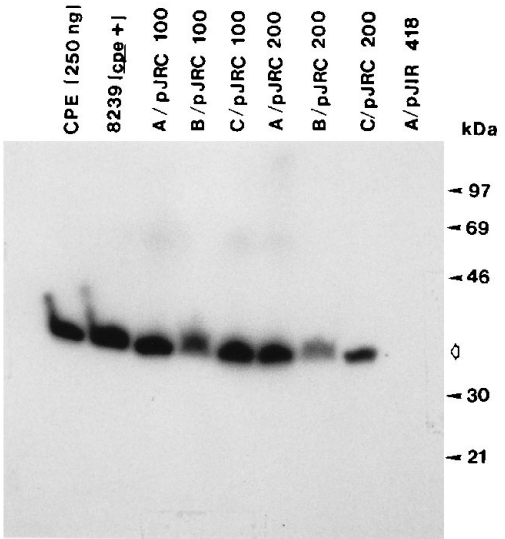

FIG. 3. Western immunoblot analysis of sporulating *C. perfringens* lysates. Lysates were analyzed for CPE expression by immunoblot analysis with CPE antiserum and ^{125}I-protein A, as described in Materials and Methods. Each lane contains 1 to 80 μl of lysate from sporulating MDS cultures of strain NCTC 8239 (8239 [*cpe*$^+$]) (15 μl); strains ATCC 3624, PS49, and CN 5383 transformed with pJRC100 (A/pJRC100 [1 μl], B/pJRC100 [80 μl], and C/pJRC100 [1 μl], respectively); strains ATCC 3624, PS49, and CN 5383 transformed with pJRC200 (A/pJRC200 [1 μl], B/pJRC200 [80 μl], and C/pJRC200 [2 μl], respectively); or strain ATCC 3624 transformed with pJIR418 (A/pJIR418) (80 μl). The open arrow indicates the migration of purified CPE stained by Coomassie blue (not shown).

TABLE 2. Quantitation of CPE expression, growth, and sporulation by *C. perfringens* transformants grown in MDS sporulation medium[a]

Culture	CPE expression (μg/ml)	Total cells/ml[b] (10^7)	Spores/ml (10^7)
NCTC 8239	15 ± 1	13 ± 2	9 ± 1
ATCC 3624 (type A)	0	12 ± 2	5 ± 1
ATCC 3624/pJIR418	0	18 ± 4	6 ± 3
ATCC 3624/pJRC100	96 ± 5	16 ± 3	6 ± 2
ATCC 3624/pJRC200	92 ± 6	19 ± 5	6 ± 2
PS49 (type B)	0	27 ± 2	0.4 ± 0.3
PS49/pJRC100	3 ± 1	26 ± 2	0.3 ± 0.1
PS49/pJRC200	2 ± 1	34 ± 8	0.4 ± 0.1
CN 5383 (type C)	0	10 ± 1	7 ± 1
CN 5383/pJRC100	90 ± 11	14 ± 5	7 ± 3
CN 5383/pJRC200	49 ± 5	10 ± 4	4 ± 2

[a] The means ± standard deviations results shown are based on at least three independent determinations for each experimental parameter for each culture.
[b] Sum of vegetative cells and sporulating cells present in each culture.

occurred within the 1.6-kb *cpe* insert present in any of these three pJRC200 transformants.

CPE Western immunoblot analyses (Fig. 2 and 3) demonstrated that all three pJRC200 transformants express CPE in a sporulation-associated pattern similar to CPE expression by both naturally enterotoxigenic *C. perfringens* food poisoning strains and our pJRC100 transformants. Specifically, none of the three pJRC200 transformants produced detectable levels of CPE during vegetative growth (Fig. 2), while all three transformants produced CPE when sporulating (Fig. 3). These differences in CPE expression do not appear to be attributable to differences in growth, since there was less than a two- to threefold difference in the amount of cell protein present per milliliter of vegetative or sporulating culture of any of these three pJRC200 transformants.

Comparison of CPE expression levels in pJRC100 and pJRC200 transformants. While the CPE Western immunoblot results shown in Fig. 3 demonstrate that all six pJRC100 and pJRC200 transformants tested express CPE in a sporulation-associated manner, they also suggest that there may be some differences in CPE expression levels among the transformants. To compare CPE expression levels in the various pJRC100 and pJRC200 transformants, a quantitative CPE Western immunoblot study was performed.

These quantitative Western blot comparisons (Table 2) confirmed that considerable (~50-fold) variation in CPE expression levels occurs when the transformants are grown in MDS sporulation medium. As shown in Table 2, the PS49 transformants consistently produced significantly smaller amounts of CPE than were produced by the ATCC 3624 or CN 5383 transformants or by the naturally enterotoxigenic NCTC 8239 strain. It is also noteworthy that, at least under the experimental conditions used for these analyses, both the ATCC 3624 and CN 5383 transformants produced even more CPE than NCTC 8239. Finally, these results also demonstrate that less than a twofold variation in CPE expression occurs between pJRC100 and pJRC200 transformants of the same *C. perfringens* strain; i.e., pJRC200 transformants given only the 1.6-kb insert produce CPE about as well as pJRC100 transformants receiving the entire 5.7-kb *Xba*I fragment.

The basis for the differences in CPE expression levels revealed by these quantitative Western immunoblot studies was investigated. Since even sporulating cultures of *C. perfringens* contain a mixture of vegetative and sporulating cells, microscopic techniques were used to examine more precisely the possible influence of either total growth or sporulation on differences in CPE expression levels between the transformants. Comparison of the cumulative numbers of both vegetative and sporulating cells present per milliliter of MDS cultures (i.e., total growth) for each transformant (Table 2) indicated that (i) introduction of either pJRC100 or pJRC200 into ATCC 3624, PS49, or CN 5383 does not significantly affect the total growth of any transformant relative to that of its nontransformed parent, (ii) there is, at most, a two- to threefold difference in total cell numbers per milliliter between any MDS cultures (a result consistent with our cell protein determinations for lysates from these cultures [data not shown]), and (iii) no positive correlation appears to exist between the overall growth of a transformant in MDS medium and the amount of CPE expressed by that transformant (e.g., the PS49 transformants, which produced low levels of CPE, grew better than any other transformants). These studies also indicated that the introduction of either pJRC100 or pJRC200 into ATCC 3624, PS49, or CN 5383 does not significantly affect the ability of these bacteria to sporulate. Further, these analyses appear to support a direct positive linkage between sporulation levels and the amount of CPE present in each culture. For example, there were significantly fewer sporulating cells in MDS cultures of the PS49 transformants, which produce low levels of CPE, than were present in MDS cultures of any other transformants. Similarly, there appears to be a positive correlation between the slightly stronger CPE expression noted for CN 5383 (pJRC100) relative to CN 5383(pJRC200) and the ca. twofold-higher sporulation levels of the CN 5383(pJRC100) transformant (Table 2). A linkage between CPE expression levels and sporulation was further supported by observations (data not shown) indicating that CN 5383(pJRC100), which sporulates and produces large amounts of CPE in MDS medium, does not sporulate well or produce CPE in raffinose-caffeine MDS sporulation medium (14), even though this transformant grew equally well in this other sporulation medium (it should be noted that *C. perfringens* isolates are known to exhibit significant strain-dependent variations in sporulation efficiency in different sporulation media [14]).

A close inspection of the results in Table 2 suggests that differences in sporulation levels may not be the only factor affecting CPE expression levels in our Western immunoblot studies. For example, although both the ATCC 3624(pJRC100) and ATCC 3624(pJRC200) transformants produced only about two-thirds as many sporulating cells when grown in modified Duncan-Strong medium as did the naturally enterotoxigenic NCTC 8239 strain, these transformants were still able to produce severalfold more CPE than NCTC 8239 could under these same growth conditions.

To investigate whether the higher CPE expression levels noted for the ATCC 3624 (and CN 5383) transformants relative to NCTC 8239 might involve a *cpe* gene dosage effect, the plasmid copy number was determined for each transformant. When grown in MDS medium, each ATCC 3624, PS49, or CN 5383 transformant was found to carry ca. two to four copies of pJRC100 or pJRC200; i.e., while there were no reproducible differences in plasmid copy number among any of the transformants, the presence of multiple (although still few) copies of the *cpe* gene in these transformants is consistent with a gene dosage phenomenon contributing to the CPE expression levels noted for the transformants. Moreover, these results indicated that the lower levels of CPE expression by PS49 transformants compared with ATCC 3624 or CN 5383 transformants do not

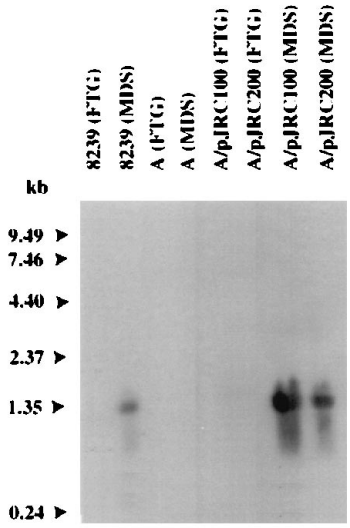

FIG. 4. Northern analysis of mRNA transcripts expressed during vegetative or sporulating growth of *C. perfringens*. Total cellular RNA isolated from vegetative (FTG) or sporulating (MDS) cultures of *C. perfringens* was subjected to Northern analysis (as described in Materials and Methods) with a 639-bp DIG-labeled *cpe*-specific DNA probe. Each lane contains 15 μg of RNA from either NCTC 8239 (8239), ATCC 3624 (A), or ATCC 3624 transformed with pJRC100 (A/pJRC100) or pJRC200 (A/pJRC200). Molecular sizes of the RNA markers are given in on the left.

appear to be explainable by the PS49 transformants maintaining lower copy numbers of pJRC100 or pJRC200.

Finally, to assess whether the sporulation-associated pattern of CPE expression noted for the pJRC100 and pJRC200 *C. perfringens* transformants could result from the presence of more copies of pJRC100 or pJRC200 per cell during sporulation than during vegetative growth, a study was performed to compare plasmid copy numbers in MDS and vegetative FTG cultures of each ATCC 3624, PS49, or CN 5383 transformant. This analysis determined that FTG cultures of all transformants also carried two to four copies of pJRC100 or pJRC200; i.e., no significant differences in plasmid copy number between vegetative and sporulating cultures of any transformant were detected.

Comparative Northern analysis of NCTC 8239 and pJRC100 and pJRC200 transformants. Having demonstrated sporulation-associated CPE expression by our transformants, it became of interest to compare transcription of *cpe* mRNA in the pJRC100 and pJRC200 *C. perfringens* transformants with that in the naturally enterotoxigenic strain *C. perfringens* NCTC 8239 by Northern analysis. As shown in Fig. 4, our *cpe*-specific probe hybridized to a single ~1.2-kb species present in RNA samples extracted from a sporulating MDS culture of NCTC 8239. However, this probe showed no hybridization to RNA samples extracted from vegetative FTG cultures of NCTC 8239 (Fig. 4), even though ethidium bromide staining (data not shown) confirmed that equivalent amounts of RNA samples from both FTG and MDS cultures of NCTC 8239 had been electrophoresed; i.e., these Northern results appear to be consistent with the transcription of *cpe* mRNA being a sporulation-associated event in naturally enterotoxigenic *C. perfringens* food poisoning strains.

As would be expected from our Southern results (Fig. 1) indicating that these strains do not carry the *cpe* gene naturally, there was no *cpe* probe hybridization to RNA samples extracted from FTG or MDS cultures of nontransformed ATCC 3624, PS49, or CN 5383 (Fig. 4 and data not shown). Similarly, the *cpe* probe did not hybridize to RNA samples extracted from MDS or FTG cultures of ATCC 3624(pJIR418) (data not shown). However, when RNA extracted from sporulating MDS cultures of either ATCC 3624(pJRC100), ATCC 3624 (pJRC200), CN 5383(pJRC100), or CN 5383(pJRC200) was subjected to *cpe* mRNA Northern analysis, a single 1.2-kb band was again visualized (Fig. 4 and data not shown). This species was not detected (Fig. 4 and data not shown) in RNA samples extracted from FTG cultures of these transformants. No *cpe* probe reactivity could be detected for RNA extracted from MDS (or FTG) cultures of PS49(pJRC100) or PS49 (pJRC200), possibly because these strains produced too little *cpe* message to allow detection, as would be consistent with Western blot results indicating low-level CPE expression by sporulating PS49 transformants (Fig. 3).

Over three experimental repetitions, the 1.2-kb *cpe* probe-reactive species was reproducibly more abundant in RNA samples extracted from the pJRC100 or pJRC200 transformants of either ATCC 3624 or CN 5383 than in RNA samples extracted from NCTC 8239. This suggests that there is a positive correlation between *cpe* message levels and the amount of CPE produced by a *C. perfringens* cell and that increased transcription and/or message stability may be involved, at least in part, in the higher CPE expression levels noted for the pJRC100 and pJRC200 transformants of ATCC 3624 and CN 5383 compared with NCTC 8239. There were no consistent differences detected in the abundance of the 1.2 kb species in RNA samples extracted from MDS cultures of pJRC100 and pJRC200 transformants of ATCC 3624 or CN 5383; i.e., the apparently greater abundance of the 1.2-kb species in the RNA sample extracted from MDS cultures of ATCC 3624(pJRC100) than in that from ATCC 3624(pJRC200) that is visible in the representative blot shown in Fig. 4 was not reproducible over three repetitions of this experiment.

To confirm that the 1.2-kb species detected on Northern blots represented an RNA species, it was shown that pretreatment of RNA samples from MDS cultures of NCTC 8239, ATCC 3624(pJRC100), or ATCC 3624(pJRC200) with DNase-free RNase resulted in the complete disappearance of the 1.2-kb species from Northern blots, while pretreatment of these same samples with RNase-free DNase did not affect the presence of the 1.2-kb species on Northern blots (data not shown).

CPE expression by pJRC100 and pJRC200 *E. coli* transformants. Given the traditional association between CPE expression and sporulation in *C. perfringens* food poisoning isolates, it was somewhat surprising when two *E. coli* DH5α transformants, named A2 and C2, were recently observed (9) to express some CPE upon transformation with identically prepared pUC19-based recombinant plasmid pA2 or pC2 (see Table 1). Since these plasmids contain the same 5.7-kb *cpe*-containing *Xba*I insert used for construction of pJRC100, we decided to ask whether *E. coli* DH5α would also express CPE after transformation with our pJIR418-based pJRC100 (or pJRC200) constructs, i.e., whether the plasmid vector used to carry the *cpe*-containing insert affects CPE expression by *E. coli.*

To address this, *E. coli* DH5α cells were transformed with pJRC100 or pJRC200, and putative transformants were then isolated on chloramphenicol-containing medium. The identities of these transformants were confirmed by Southern analysis, which demonstrated (data not shown) the presence of an ~13-kb *cpe*-containing plasmid, with a 5.7-kb *cpe*-containing *Xba*I insert, in a transformant named DH5α(pJRC100) and the presence of a 9-kb *cpe*-containing plasmid, with a 1.6-kb *cpe*-containing *Eco*RI-*Xba*I fragment, in a transformant named DH5α(pJRC200). The ability of these two transformants to

TABLE 3. Plasmid copy number in *E. coli* DH5α

Plasmid	Copy no.[a]	CPE[b]
pUC19	330	−
pA2	820	+
pC2	830	+
pJIR418	390	−
pJRC100	45	−
pJRC200	81	−

[a] Mean from at least three experiments.
[b] +, CPE expressed when plasmid is introduced into DH5α; −, CPE not expressed when plasmid is introduced into DH5α.

express CPE was then compared with that of pA2 or pC2 *E. coli* DH5α transformants. CPE expression could be detected in culture lysates from DH5α clones transformed with either pA2 or pC2 (Fig. 2), but, despite maximal sample loading, no CPE expression was detected in culture lysates from DH5α transformed with pJRC100 or pJRC200 (or with control plasmid pJIR418 or pC1 [Table 1]). These differences in CPE expression do not appear to be explainable by differences in the growth of these transformants, since all cultures contained approximately the same amount of cell protein per milliliter (data not shown).

One possible explanation for the CPE expression differences observed between the pA2 or pC2 and the pJRC100 or pJRC200 transformants (Fig. 2) could be that DH5α simply carries more copies of the pUC19-based pA2 or pC2 plasmid than of the pJIR418-based pJRC100 or pJRC200 plasmid. Plasmid copy number analysis (Table 3) confirms that DH5α does carry pJRC100 at a 20-fold-lower copy number and pJRC200 at a 10-fold-lower copy number compared with the copy number of either pA2 or pC2, a result which is consistent with the involvement of a strong gene dosage effect in CPE expression by *E. coli.*

DISCUSSION

The sporulation-associated expression of CPE in *C. perfringens* food poisoning isolates strongly suggests the involvement of regulatory factors in CPE expression, but little research has been conducted on this topic to date. To initiate such studies, we have asked in the current work whether naturally *cpe*-negative *C. perfringens* cells, which apparently represent ~95% of the total *C. perfringens* population (14, 28), would express CPE if they were provided, on a low-copy-number plasmid, the *cpe* gene of *C. perfringens* food poisoning isolate NCTC 8239. Our results indicate not only that these transformants express CPE, even when provided a plasmid (pJRC200) with a *C. perfringens* DNA insert containing only the *cpe* ORF (but no other ORFs), but also that this expression appeared to be highly regulated; i.e., CPE expression by the transformants closely mimicked the sporulation-associated pattern of CPE expression exhibited by naturally enterotoxigenic *C. perfringens* food poisoning isolates.

These results provide several interesting observations. First, it was particularly noteworthy that even the type B *C. perfringens* transformants expressed CPE in a sporulation-associated manner, since, to our knowledge, there have not yet been reliable reports of naturally enterotoxigenic type B *C. perfringens* isolates. Second, it was interesting that the pJRC100 and pJRC200 transformants of both *C. perfringens* ATCC 3624 and CN 5383 consistently expressed severalfold more CPE than naturally enterotoxigenic *C. perfringens* NCTC 8239, especially if expression levels were corrected for sporulation differences. Since pJRC100 and pJRC200 are maintained at two to four copies per transformed *C. perfringens* cell while food poisoning strains such as NCTC 8239 are thought to carry only a single copy of the *cpe* gene (5), these expression results suggest that even though sporulation-associated CPE expression is already very strong in *C. perfringens* strains carrying a single copy of *cpe* (in which CPE can represent >10 to 20% of the total protein present in the sporulating cells of a food poisoning isolate), sporulating *C. perfringens* cells have the capacity to produce even more CPE if they are provided with multiple copies of the enterotoxin gene. Third, considering recent evidence (5–7) suggesting that the *cpe* gene, even when localized to the *C. perfringens* chromosome, is present on a mobile genetic element, it was notable that less-than-twofold differences in CPE expression were observed between pJRC100 transformants (which were provided with >2 kb of both upstream and downstream DNA flanking the *cpe* gene) and pJRC200 transformants (which were provided with *cpe* but no other flanking ORFs) of the same host strain. Presuming it unlikely that all three *cpe*-negative host strains used in our study would harbor a mobile genetic element similar to the *cpe* mobile genetic element present in food poisoning isolates except for the omission of the *cpe* gene, these results, in combination with the results indicating that the transformants produce even more CPE than NCTC 8239, strongly suggest that other ORFs present on the putative *cpe*-containing mobile genetic element in food poisoning isolates are not essential for CPE expression.

Carrying the interpretation of our pJRC200 expression data further, the observation that the pJRC200 transformants of all three naturally *cpe*-negative *C. perfringens* host strains were able to express CPE in a sporulation-associated manner also suggests that many (if not all) *C. perfringens* isolates (including the vast majority of *C. perfringens* isolates that do not carry the *cpe* gene) are routinely producing at least some of the factors that naturally enterotoxigenic *C. perfringens* food poisoning isolates use to regulate CPE expression. It seems unlikely that *cpe*-negative *C. perfringens* would bother producing factors involved in CPE regulation unless these same factors were also involved in regulating other *C. perfringens* genes.

Given the sporulation-associated nature of CPE expression, it would be helpful if analogies could be drawn from the elegant work on the regulation of gene expression by members of the endospore-forming genus *Bacillus*. Unfortunately, the lack of basic research comparing regulatory factors in *Clostridium* and *Bacillus* spp. precludes extensive discussion of possible regulatory models for CPE expression at this time. The only information currently available in this regard is the recent identification of putative Hpr-binding sequences, which exactly match consensus *Bacillus subtilis* Hpr-binding sequences, in regions of *C. perfringens* DNA lying immediately upstream and downstream of the *cpe* gene. Since it is known that, in *B. subtilis*, Hpr is a transition state regulator that represses expression levels of some proteins during the exponential phase of vegetative growth, the discovery of these Hpr-binding sequences in DNA regions flanking the *cpe* gene in *C. perfringens* has led to the proposal that a *C. perfringens* Hpr homolog may help regulate CPE expression (5). If a regulatory system involving Hpr was found to be involved in CPE expression in *C. perfringens*, the recent demonstration (5) that DNA from most, if not all, *C. perfringens* isolates (including several *cpe*-negative isolates) will specifically hybridize an *hpr* probe could help explain why all three of our naturally *cpe*-negative transformants were able to express CPE in a regulated manner. Hpr involvement in the regulation of CPE expression also appears to be consistent with our hypothesis that some regulatory gene(s) modulating CPE expression also helps regulate oth-

er *C. perfringens* genes, since Hpr has been shown to regulate a number of different genes in *B. subtilis* (26). Therefore, it appears to be important that future studies determine whether an Hpr homolog is actually expressed by *C. perfringens* and, if so, to directly evaluate the proposal (5) that this protein is involved in the regulation of CPE expression.

Even if the involvement of an Hpr-like system in CPE expression is eventually demonstrated, this need not preclude the possible participation of other regulatory systems in modulating CPE expression levels. In this regard, it may be noteworthy that we were unable to detect CPE expression by *E. coli* transformants carrying even 50 to 100 copies of the *cpe* gene on pJRC100 or pJRC200. Since *E. coli* RNA polymerase has been shown to recognize the promoters of several vegetatively expressed *C. perfringens* genes (11, 22) and since several vegetatively expressed *C. perfringens* toxins appear to be well expressed from clostridial promoters in *E. coli* (12, 22), the failure of the pJRC100 and pJRC200 *E. coli* transformants to express CPE appears to be consistent with either (i) *E. coli* producing some regulatory factor capable of repressing transcription of the *cpe* gene (this putative *E. coli* repressor would presumably be distinct from the Hpr-like system discussed above) or, perhaps more likely, (ii) CPE expression in *C. perfringens* involving positive regulation by a sporulation-associated regulatory factor(s) (e.g., a variant sigma factor) that is not produced by *E. coli* (in this case, the small amounts of CPE produced by *E. coli* transformed with *cpe*-containing pUC19 derivatives could be resulting from a small amount of "leaky" transcription occurring from the very large number of *cpe* genes present in these transformants). Clearly, studies of CPE expression regulation remain in their infancy, and much work remains to be done to clarify what may ultimately prove to be a quite complicated regulatory system.

By demonstrating that sporulating cells of the naturally enterotoxigenic *C. perfringens* strain NCTC 8239 appear to contain a single *cpe* mRNA species of 1.2 kb, our Northern results are consistent with *cpe* mRNA being transcribed as a monocistronic message. This finding is consistent with previous suggestions that transcription of the 0.96-kb *cpe* ORF starts at one or more promoters located ~50 to 200 bp upstream of the *cpe* initiation codon (18) and terminates at a putative termination loop located ~40 bp downstream of the *cpe* termination codon (9). Because the precise identity of the transcriptional start and termination sites for the *cpe* message remain ambiguous, additional studies are clearly required to confirm these putative relationships. However, it is noteworthy that an identically comigrating 1.2-kb *cpe* mRNA species was also detected in the pJRC100 and pJRC200 transformants of ATCC 3624 and CN 5383. These results are consistent with *cpe* mRNA transcription in these *C. perfringens* transformants involving promoter usage and termination sequences similar to those that occur in naturally enterotoxigenic *C. perfringens* isolates. Unfortunately, we were unable to detect *cpe* message in the *E. coli* C2 or A2 transformants, which produce very low levels of CPE, by Northern analysis, so the sizes of *cpe* mRNAs made by *C. perfringens* and *E. coli* were not compared in the current study; i.e., it remains to be determined whether *cpe* transcription is being driven from the same promoter sequences in recombinant *E. coli* as in *C. perfringens.*

Northern blot detection of the 1.2-kb *cpe* mRNA species in sporulating, but not vegetative, cultures of NCTC 8239 (and the pJRC100 and pJRC200 ATCC 3624 or CN 5383 transformants) is consistent with CPE expression involving regulation at the transcriptional level, supporting previous suggestions provided by some recent RNA slot blot studies (18). As Melville et al. also noted in their study (18), these results do not preclude the possibility that posttranscriptional effects may also contribute to differences in CPE expression levels between various isolates. Since posttranscriptional factors such as differences in mRNA stability could also play important roles in determining how much CPE an isolate produces, it will be important to perform detailed kinetic analyses of transcriptional versus posttranscriptional effects on CPE expression levels in selected *C. perfringens* isolates.

Besides reporting the apparent first application of Northern blot analysis to sporulation-associated clostridial virulence factors, this paper also reports the successful preparation of type B *C. perfringens* transformants, which is notable given previous unsuccessful attempts at electroporating plasmids into type B *C. perfringens* isolates (2). However, our type B PS49 transformants were difficult to obtain, with their transformation frequency ($\sim 10^{-6}$ transformants per viable cell) being considerably lower than the 10^{-3} to 10^{-4} transformants per viable cell obtained with ATCC 3624 (a frequency matching previous electroporation results for this strain [1, 2]) or the 10^{-5} transformants per viable cell obtained with CN 5383. Since attempts to transform two other type B *C. perfringens* strains by the same protocol used for PS49 proved to be unsuccessful during our studies, it remains unclear why some *C. perfringens* strains, particularly type B strains, appear to be so refractile to transformation by electroporation.

Finally, three different *C. perfringens* strains have been successfully transformed by electroporation in the current study, with the resultant transformants used to examine the expression of a single clostridial virulence factor. This is noteworthy considering that nearly all recent studies of *C. perfringens* virulence factor expression have utilized only the easily transformable strain *C. perfringens* 13 (or its derivatives). By introducing the same virulence factor gene into several *C. perfringens* strains, the current work establishes a precedent for comparing virulence factor expression in multiple *C. perfringens* host backgrounds, an approach that may identify strain-related differences in clostridial virulence factor expression.

ACKNOWLEDGMENTS

The first two authors contributed equally to this work.

We thank Julian Rood for providing pJIR418, Ron Labbe for providing strain PS49, Ewa Wieckowski for assistance with computer graphics and Marilyn Williams for secretarial assistance.

This work was supported by Public Health Service grant AI 19844-13.

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Editor: A. O'Brien