

Organization of the Plasmid *cpe* Locus in *Clostridium perfringens* Type A Isolates

Kazuaki Miyamoto,^{1,2} Ganes Chakrabarti,¹ Yosiharu Morino,² and Bruce A. McClane^{1*}

Department of Molecular Genetics and Biochemistry, University of Pittsburgh School of Medicine, Pittsburgh, Pennsylvania 15261,¹ and Department of Microbiology, Wakayama Medical College, Wakayama, Japan 641-00122²

Received 14 January 2002/Returned for modification 19 February 2002/Accepted 14 May 2002

Clostridium perfringens type A isolates causing food poisoning have a chromosomal enterotoxin gene (*cpe*), while *C. perfringens* type A isolates responsible for non-food-borne human gastrointestinal diseases carry a plasmid *cpe* gene. In the present study, the plasmid *cpe* locus of the type A non-food-borne-disease isolate F4969 was sequenced to design primers and probes for comparative PCR and Southern blot studies of the *cpe* locus in other type A isolates. Those analyses determined that the region upstream of the plasmid *cpe* gene is highly conserved among type A isolates carrying a *cpe* plasmid. The organization of the type A plasmid *cpe* locus was also found to be unique, as it contains IS1469 sequences located similarly to those in the chromosomal *cpe* locus but lacks the IS1470 sequences found upstream of IS1469 in the chromosomal *cpe* locus. Instead of those upstream IS1470 sequences, a partial open reading frame potentially encoding cytosine methylase (*dcm*) was identified upstream of IS1469 in the plasmid *cpe* locus of all type A isolates tested. Similar *dcm* sequences were also detected in several *cpe*-negative *C. perfringens* isolates carrying plasmids but not in type A isolates carrying a chromosomal *cpe* gene. Contrary to previous reports, sequences homologous to IS1470, rather than IS1151, were found downstream of the plasmid *cpe* gene in most type A isolates tested. Those IS1470-like sequences reside in about the same position but are oppositely oriented and defective relative to the IS1470 sequences found downstream of the chromosomal *cpe* gene. Collectively, these and previous results suggest that the *cpe* plasmid of many type A isolates originated from integration of a *cpe*-containing genetic element near the *dcm* sequences of a *C. perfringens* plasmid. The similarity of the plasmid *cpe* locus in many type A isolates is consistent with horizontal transfer of a common *cpe* plasmid among *C. perfringens* type A strains.

The gram-positive, anaerobic pathogen *Clostridium perfringens* is classified into five biotypes (A to E), based upon production of alpha, beta, epsilon, and iota toxins (14). Approximately 5% of *C. perfringens* isolates, mostly belonging to type A, produce another important toxin named *C. perfringens* enterotoxin (CPE) (12–14, 22). CPE-producing type A isolates are major human gastrointestinal (GI) pathogens, causing *C. perfringens* type A food poisoning (14) and such non-food-borne human GI diseases as antibiotic-associated diarrhea and sporadic diarrhea (15, 16). Enterotoxigenic type A isolates may also be responsible for some veterinary GI illnesses (21). Recent studies with *cpe* knockout mutants confirmed that CPE expression is necessary for the enteric virulence of enterotoxigenic *C. perfringens* type A isolates (20).

The gene encoding *C. perfringens* enterotoxin (*cpe*) can be located on either the chromosome or large plasmids (2–8, 23). Most (if not all) enterotoxigenic *C. perfringens* type A isolates associated with food poisoning carry their *cpe* genes on the chromosome (2–8, 23). Sequencing and Southern blot studies demonstrated that the chromosomal *cpe* gene of type A food poisoning strain NCTC8239 (and, apparently, other *C. perfringens* type A food poisoning strains) is closely associated with insertion sequences (2, 3, 5, 8). In NCTC8239, those *cpe*-associated insertion sequences include a single IS1469 sequence present ~1.3 kbp upstream of the *cpe* gene and a pair

of IS1470 sequences residing ~3 kb upstream and ~1.2 kbp downstream of the *cpe* gene (5, 8). Based upon this arrangement (see Fig. 1), it was proposed that the chromosomal *cpe* gene of NCTC8239 (and, by extension, other type A food poisoning isolates) is located on a 6.3-kbp transposon with terminal IS1470 elements (5). More recent results (2) suggest that this putative transposon, named Tn5565, may have several circular intermediate forms. However, actual movement of Tn5565 has not yet been demonstrated.

Interestingly, the *cpe* gene is plasmid localized in most (if not all) enterotoxigenic type A isolates obtained from either CPE-associated non-food-borne human GI disease or veterinary sources (7, 8, 23). The *cpe* plasmids of those type A isolates are large (~100 kbp) and present at low copy numbers (7, 8, 20). The *cpe* plasmid of the type A human sporadic diarrhea isolate F4969 was recently shown to be transferable, via conjugation, to *cpe*-negative *C. perfringens* type A strains (4).

The plasmid *cpe* locus of type A isolates has not yet been sequenced or otherwise studied in comparable detail as the chromosomal *cpe* locus of NCTC8239. To our knowledge, the only information currently available regarding the plasmid *cpe* locus of type A isolates was provided by Southern blot analyses (8) of just three such isolates (only one of which, isolate F3686, was associated with human non-food-borne GI disease). Those limited studies revealed that, as for type A food poisoning isolate NCTC8239, which carries a chromosomal *cpe* gene, IS1469 sequences appear to be present directly upstream of the plasmid *cpe* gene in the three initially examined type A isolates. However, no IS1470 sequences were detected near the plasmid *cpe* gene of those isolates. Instead, two of the isolates (includ-

* Corresponding author. Mailing address: Department of Molecular Genetics and Biochemistry, E1240 BSTWR, University of Pittsburgh School of Medicine, Pittsburgh, PA 15261. Phone: (412) 648-9022. Fax: (412) 624-1401. E-mail: bamcc@pitt.edu.

TABLE 1. *C. perfringens* isolates used in this study

Isolate group	Strain	Type	Location of <i>cpe</i>	Date and source	Reference
Food poisoning isolates	NCTC10239	A	Chromosome	1950s, Europe	7
	NCTC8239	A	Chromosome	1950s, Europe	7
	NCTC8798	A	Chromosome	1950s, Europe	7
	537-5	A	Chromosome	Late 1990s, North America	23
	538-1	A	Chromosome	Late 1990s, North America	23
Non-food-borne human GI disease isolates	F4969	A	Plasmid	Early 1990s, Europe, sporadic diarrhea	7
	F4013	A	Plasmid	Early 1990s, Europe, sporadic diarrhea	7
	F5603	A	Plasmid	Early 1990s, Europe, sporadic diarrhea	7
	B40	A	Plasmid	1980s, Europe, antibiotic-associated diarrhea	7
	Newbury16 (NB 16)	A	Plasmid	1980s, Europe, antibiotic-associated diarrhea	7
	T34058	A	Plasmid	Late 1990s, North America, antibiotic-associated diarrhea	23
	M26413	A	Plasmid	Late 1990s, North America, antibiotic-associated diarrhea	23
	W30554	A	Plasmid	Late 1990s, North America, antibiotic-associated diarrhea	23
Normal human fecal isolates	MR1-1	A	<i>cpe</i> negative	2000, Japan	This study
	MR2-2	A	<i>cpe</i> negative	2000, Japan	This study
	MR2-4	A	Plasmid	2000, Japan	This study
Isolates from veterinary sources	157	A	Plasmid	Early 1990s, North America, canine peritoneal fluid	7
	452	A	Plasmid	Early 1990s, North America, canine diarrhea	7
	315455 N4	A	Plasmid	Late 1990s, North America, canine diarrhea	This study
	316366 D3	A	Plasmid	Late 1990s, North America, canine diarrhea	This study
	297442	A	<i>cpe</i> negative	Late 1990s, North America, canine diarrhea	This study
Other isolates	NCTC8533	B	<i>cpe</i> negative		23
	CN5383	C	<i>cpe</i> negative		23
	PS52	D	<i>cpe</i> negative		23
	853	E	Plasmid ^a		23

^a Like most type E isolates (1), 853 carries silent *cpe* sequences containing nonsense and missense mutations in the *cpe* ORF.

ing the human non-food-borne-GI-disease isolate F3686) apparently carry IS1151 sequences ~260 bp downstream of their plasmid *cpe* gene.

The limited information now available regarding the organization of the plasmid *cpe* locus suggests that considerable variation exists between the organization of the chromosomal *cpe* locus and the plasmid *cpe* locus of *C. perfringens* type A isolates. To better address that possibility and to assess the diversity of the plasmid *cpe* locus in different type A isolates, we first cloned and sequenced the plasmid *cpe* locus of human sporadic diarrhea strain F4969, a well-studied type A isolate (4, 6, 7, 20). The sequencing results obtained for the F4969 plasmid *cpe* locus were then used to prepare primers and probes for conducting PCR and Southern blot analyses to further explore the organization of the *cpe* locus in other type A isolates, including a number of plasmid *cpe* isolates originating from various sources (including non-food-borne human GI diseases).

MATERIALS AND METHODS

Bacterial strains. The toxin genotype, *cpe* genotype, and source of each *C. perfringens* isolate examined in this study are listed in Table 1. Our study used six previously uncharacterized *C. perfringens* isolates, including normal human fecal isolates (MR1-1, MR2-2, and MR2-4) and veterinary isolates (316366 D3, 315455 N4, and 297442). These six new isolates were first subjected to multiplex PCR analysis (23), which confirmed their identity as type A isolates and revealed

whether they carried *cpe* sequences. As indicated in Table 1, the three new type A isolates identified as *cpe* positive by multiplex PCR were then subjected to *cpe* restriction fragment length polymorphism analysis (23) in order to determine whether they carried a chromosomal or plasmid *cpe* gene.

Escherichia coli strain DH5 α was grown in tryptic soy broth medium and used for transformation experiments.

DNA isolation from *C. perfringens* isolates. *C. perfringens* isolates were cultured for 6 to 8 h at 37°C in TGY medium, as described previously (20). Total DNA was extracted from those TGY cultures using a previously described method (23). Plasmid DNA was isolated from TGY cultures by the method of Roberts et al. (18), except that RNase treatment was omitted.

Cloning of *Clal* and *XbaI* fragments containing the plasmid *cpe* gene of *C. perfringens* type A isolate F4969. Plasmid DNA from isolate F4969 was digested overnight at 37°C with either *Clal* or *XbaI* (Roche). That digested plasmid DNA was electrophoresed on 1% agarose gels, and DNA fragments of ~5 kbp (for *Clal*-digested plasmid DNA) or ~9 kbp (for *XbaI*-digested plasmid DNA) were then extracted with a DNA purification kit (Bio-Rad). A pBlueScript vector was digested with either *Clal* or *XbaI* and then treated with calf intestinal phosphatase (Roche). That dephosphorylated plasmid was ligated, for 16 h at 16°C, with either the isolated ~5-kbp *Clal* or ~9-kbp *XbaI* fragments of F4969 plasmid DNA (19). Ligated plasmids were then transformed into *E. coli* DH5 α host cells by using standard techniques (19).

DNA sequencing of *E. coli* transformants containing F4969 DNA. Southern blotting with a *cpe*-specific probe (7, 20, 23) identified several *E. coli* transformants carrying F4969 *cpe* sequences (data not shown). Two transformant clones, named KCpeFC5-1 (containing an ~5-kbp *Clal* insert of F4969 plasmid DNA) and KCpeFX28 (containing an ~9-kbp *XbaI* insert of F4969 plasmid DNA), were then used to DNA sequence the plasmid *cpe* locus of isolate F4969. DNA sequencing was initially performed with universal primers for the pBlueScript

vector. Additional sequencing was performed using primers designed from those initial and subsequent sequencing results (see Fig. 1).

IS1469-*cpe* PCR. Based upon sequencing results obtained for the *cpe*-containing *Xba*I and *Cla*I fragments of KCpeFX28 and KCpeFC5-1, the following primers complementary to IS1469 and *cpe* gene sequences were designed: 5'-A AGTGAGATATTAAGGCAGC-3' (IS1469F) and 5'-CCTAATATCCAACCA TCTCC-3' (CPE-up). These primers (1 μ M final concentration) were then included in a PCR utilizing template DNA (total DNA extracted from specified *C. perfringens* type A isolates), 0.2 mM deoxynucleotide triphosphates (Promega), 2.5 mM MgCl₂, and 5 U of *Taq* polymerase (Promega). The reaction mixtures, in a total volume of 50 μ l, were placed in a thermal cycler (Techne) under the following conditions: 1 cycle at 94°C for 5 min; 33 cycles of 94°C for 30 s, 60°C for 60 s, and 68°C for 180 s; and 1 cycle at 94°C for 90 s, 60°C for 90 s, and 68°C for 10 min. The resultant PCR products were run on a 2% agarose gel and stained with ethidium bromide.

Cytosine methyltransferase gene (*dcm*) PCR. After sequencing of the *cpe*-containing *Xba*I fragment in KCpeFX28 had revealed the presence of sequences that could encode the C-terminal half of cytosine methyltransferase (*dcm* sequences), the following primer pair was designed to PCR amplify those putative *dcm* sequences: 5'-CGATAATCT AGTTACTACTAGAGAGC-3' (MET-F) and 5'-CTGGATTACAATACTATTCCCAGC-3' (MET-R). These primers were added (at a 1 μ M final concentration) to a PCR mixture utilizing template DNA (total DNA extracted from specified *C. perfringens* isolates), 0.1 mM deoxynucleotide triphosphates (Promega), 2.5 mM MgCl₂, and 2.5 U of *Taq* polymerase (Promega). The reaction mixtures, in a total volume of 50 μ l, were then placed in a thermal cycler (Techne) under the following conditions; 1 cycle at 94°C for 2 min; 38 cycles at 94°C for 30 s, 50°C for 60 s, and 68°C for 60 s; and 1 cycle at 94°C for 90 s, 50°C for 90 s, and 68°C for 7 min.

To PCR amplify the DNA region lying between *dcm* sequences and sequences located ~400 bp downstream from those *dcm* sequences in F4969, a second PCR was performed with MET-F and a primer named UP-IS (see Fig. 1) (5'-ATAT TGGCCACATTCACAGC-3'). The PCR conditions used with these primers were as described above for amplifying internal *dcm* sequences. All *dcm*-based PCR products were electrophoresed on a 2% agarose gel, which was then stained with ethidium bromide.

After agarose electrophoresis and transfer to nylon membranes (Roche), PCR products amplified with MET-F and UP-IS from selected *C. perfringens* isolates (see Fig. 6) were Southern blotted by conventional techniques (20). The blots were hybridized with a digoxigenin (DIG)-labeled PCR probe prepared (9) with a PCR DIG-labeling kit (Roche), KCpeFX28 template DNA, and MET-F and UP-IS.

Southern blotting analyses for the presence of *cpe*, *dcm* sequences, IS1470-like sequences, and IS1151-like sequences in *C. perfringens* isolates. For additional Southern blotting analyses, four more DIG-labeled probes were also generated with the PCR DIG-labeling kit. A *dcm* sequence-specific probe was prepared in a PCR using the plasmid present in KCpeFX28 as the template DNA, along with MET-F and MET-R primers. A *cpe*-specific probe was prepared in a PCR using F4969 DNA and primers 2F (5'-GGTACCTTTAGCCAATCA-3') and 5R (5'-TCCATCACCTAAGGACTG-3'), which are specific for internal *cpe* sequences. To prepare a probe for internal IS1470-like sequences, PCR was performed with the primers IS1470-likeF (5'-GATAACTACTATGGACTACC-3') and IS1470-likeR (5'-CGTATTAGACCATTATGACG-3'), with KCpeFX28 as the template DNA. Finally, a probe for internal IS1151-like sequences was prepared from F4013 DNA by using the primers IS1151-F (5'-TCCGATATCTGTACGG CTCC-3') and IS1151-R (5'-AGAGCCAATATAGATATCAGTAAG-3'), which are complementary to the previously reported (11) IS1151 sequence.

For Southern blotting analyses to detect the presence of *cpe*, *dcm* sequences, IS1470-like sequences, and IS1151-like sequences, plasmid preparations isolated from specified *C. perfringens* isolates were digested overnight with *Xba*I at 37°C. To detect the presence of *dcm* sequences in *C. perfringens* food poisoning isolates, total DNA was obtained from each isolate and digested overnight with *Xba*I at 37°C. Digested plasmid or total DNAs were electrophoresed on a 1% agarose gel (Bio-Rad) and then transferred with a vacuum blotter (Bio-Rad) onto a nylon membrane. The membranes were hybridized, as previously described (20), with one of the probes described above.

Comparative PCR analyses of the DNA region upstream of plasmid or chromosomal *cpe* genes in *C. perfringens* type A isolates. Based upon the KCpeFX28 sequencing results, the DNA region between *dcm* sequences and IS1469 was explored with a PCR using the MET-F primer and IS1469R (5'-CCGCATAC TCTTTACTTGC-3'), a primer specific for IS1469 sequences. These primers (1 μ M final concentration) were used in a PCR with template DNA (total DNA extracted from specified *C. perfringens* isolates), 0.2 mM deoxynucleotide triphosphates, and 1 μ l of Advantage 2 *Taq* polymerase (Clontech). The reaction

mixtures, in a total volume of 50 μ l, were placed in a thermal cycler (Techne) under the following conditions: 1 cycle at 94°C for 5 min; 33 cycles at 94°C for 30 s, 50°C for 60 s, and 68°C for 180 s; and 1 cycle at 94°C for 90 s, 50°C for 90 s, and 68°C for 7 min. The products were run on a 2% agarose gel and stained with ethidium bromide.

A second PCR to amplify the DNA region between *dcm* sequences and the *cpe* gene was also performed with the primers MET-F and CPE-up, along with Advantage 2 *Taq* polymerase, under the following conditions: 1 cycle at 94°C for 2 min; 34 cycles at 94°C for 30 s; 60°C for 60 s, and 68°C for 180 s; and 1 cycle at 94°C for 90 s, 60°C for 90 s, and 68°C for 7 min.

Products of both the *dcm*-IS1469 and *dcm*-*cpe* PCRs were electrophoresed on a 2% agarose gel and then stained with ethidium bromide.

Comparative PCR analysis of the DNA region downstream of plasmid versus chromosomal *cpe* genes in *C. perfringens* type A isolates. Primers complementary to sequences present in *cpe* (CPE-4.5F, 5'-CAGTCCTTAGGTGATGGA-3') or the IS1470-like sequences of F4969 (IS1470-likeR) were used to investigate the DNA region downstream of the plasmid or chromosomal *cpe* gene in *C. perfringens* type A isolates. This PCR utilized total DNA extracted from specified type A isolates as the template and also included each primer (1 μ M final concentration), 0.2 mM deoxynucleotide triphosphates (Promega), and 1 μ l of Advantage 2 *Taq* polymerase (Clontech). The reaction mixtures, in a total volume of 50 μ l, were placed in a thermal cycler (Techne) and amplified under the conditions described above for the *dcm*-*cpe* PCR. PCR products were run on a 2% agarose gel and stained with ethidium bromide.

A second PCR to amplify internal IS1470-like sequences was also performed with both the IS1470-likeF and -R primers. These primers were added (at a 1 μ M final concentration) to a PCR utilizing template DNA (total DNA extracted from specified *C. perfringens* isolates), 0.1 mM deoxynucleotide triphosphates (Promega), 2.5 mM MgCl₂, and 2.5 U of *Taq* polymerase. The reaction mixtures, in a total volume of 50 μ l, were then placed in a thermal cycler (Techne) under the following conditions: 1 cycle at 94°C for 2 min; 38 cycles at 94°C for 30 s, 44°C for 60 s, and 72°C for 60 s; and 1 cycle at 94°C for 90 s, 44°C for 90 s, and 72°C for 10 min.

PCR analysis for IS1151 sequences. To amplify internal IS1151 sequences, a PCR was performed with IS1151-F and -R primers, which are complementary to the previously reported (11) IS1151 sequence. These primers were used in a PCR under amplification conditions described earlier for detecting *dcm* sequences.

A final PCR assay to evaluate the location of IS1151 sequences relative to the *cpe* gene was performed using either CPE-4.5F or CPE-d1F (5'-CTAACTCAT ACCCTTGGACTC-3'), along with the IS1151-R primer, in a PCR run under conditions described above for amplifying internal IS1151 sequences.

Nucleotide sequence accession number. The sequence of the ~9-kbp *Xba*I fragment in KCpeFX28 was submitted to GenBank under accession number AF416450. The sequences obtained for the PCR products described below were submitted to GenBank under accession numbers AF506816 to -18 and AF511071.

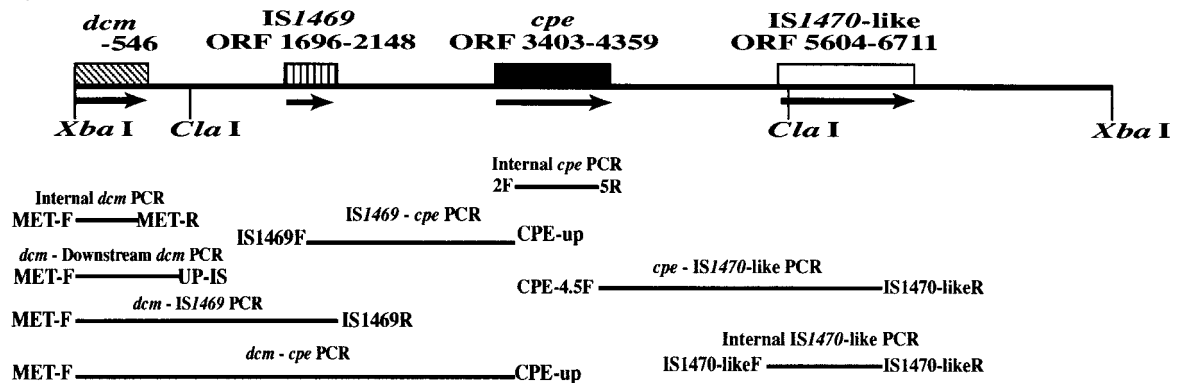
RESULTS

Cloning and sequencing of *Cla*I and *Xba*I fragments carrying the plasmid *cpe* gene of F4969, a *C. perfringens* type A human sporadic-diarrhea isolate. Initial Southern blotting results (data not shown) indicated that the plasmid *cpe* gene of type A isolate F4969 is present on an ~5-kbp *Cla*I fragment and an ~9-kbp *Xba*I fragment. Therefore, those two *cpe*-containing fragments were separately cloned into a pSK vector. The resultant plasmids were then used to transform *E. coli*, as described in Materials and Methods. Southern blotting with *cpe* probes (data not shown) confirmed the presence of *cpe* sequences in two transformants, named KCpeFC5-1 and KCpeFX28, which carry (respectively) an ~5-kbp *Cla*I fragment and an ~9-kbp *Xba*I fragment.

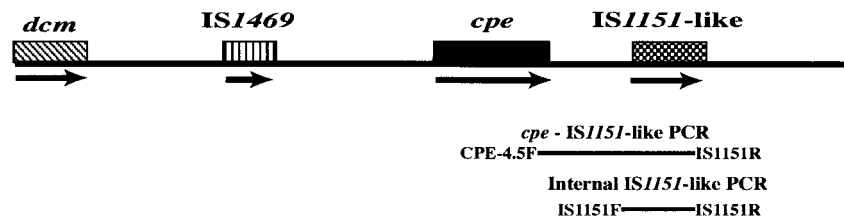
DNA sequencing of KCpeFC5-1 and KCpeFX28 confirmed a previous report (6) that the *cpe* open reading frame (ORF) of isolate F4969 is completely homologous to the chromosomal *cpe* ORF of type A food poisoning strain NCTC8239 (10). Sequences downstream or upstream of the *cpe* ORF had not yet been examined for any type A isolate carrying a *cpe* plas-

I. Plasmid *cpe* strains

(A) F4969



(B) F4013



II. Chromosomal *cpe* Strain NCTC8239



FIG. 1. Genetic organization of the *cpe* locus in *C. perfringens* type A isolates. (I) Type A isolates carrying a *cpe* plasmid, including the sequenced \sim 9-kbp *Xba*I *cpe*-containing fragment of the *cpe* plasmid from sporadic diarrhea isolate F4969 (A) and the deduced plasmid *cpe* locus of isolates F4013 (B). (II) The previously described (5) chromosomal *cpe* locus of food poisoning strain NCTC8239 is shown for comparison. Broad bars show ORFs; arrows under ORFs indicate orientation. Numbers shown for F4969 indicate nucleotide bases. Long thin bars depict the PCR products amplified with each primer pair (see the text).

mid. However, our KCpeFC5-1 and KCpeFX28 sequencing data indicate that, with the exception of a few nucleotide differences, the putative stem-loop structure located \sim 40 bp downstream of the chromosomal *cpe* stop codon in NCTC8239 DNA (10) is similarly present downstream of the plasmid *cpe* ORF of isolate F4969. As also noted in the chromosomal *cpe* locus of NCTC8239 (10), the putative stem-loop structure downstream of the F4969 *cpe* ORF is immediately followed by an oligo(T) tract, which is consistent with the idea that stem-loop-oligo(T) region functions as a rho-independent transcription terminator of *cpe* transcription in sporulating F4969 cells (10).

These sequencing analyses also revealed that, except for a few single-nucleotide differences, the downstream DNA of F4969 and NCTC8239 remain homologous for \sim 1 kbp past their *cpe* ORF. Significant downstream sequence divergence occurs beyond that conserved region (Fig. 1), with the *cpe* plasmid of F4969 having a defective ORF that shares partial homology (Fig. 2) with an *IS1470* element present, in about the same location, downstream of the NCTC8239 chromosomal *cpe* gene (5). However, relative to the *IS1470* sequences downstream of the NCTC8239 *cpe* gene (10), the *IS1470*-like ele-

ment of F4969 contains many nucleotide replacements (some introducing nonsense mutations), is oppositely oriented, and has a 84-bp deletion and a 88-bp insertion (Fig. 2).

With respect to sequences upstream of the F4969 plasmid *cpe* ORF, our KCpeFC5-1 and KCpeFX28 sequencing results revealed that the Shine-Dalgarno sequence and four putative promoters of F4969 are identical, in location and sequence, to those identified previously in the NCTC8239 chromosomal *cpe* gene (3, 5, 17, 24). However, about 250 bp upstream of the F4969 *cpe* gene, a 45-bp insertion was detected. That insertion is absent from the chromosomal *cpe* locus of NCTC8239 but completely matches an insertion found, in the same location, upstream of the chromosomal *cpe* gene of another type A food poisoning strain, NCTC10240 (17).

Further (1.3 kbp) upstream of the F4969 plasmid *cpe* gene, *IS1469* sequences were detected (Fig. 1). Except for two nucleotide changes, those insertion sequences were found to match *IS1469* sequences located, in the same position and orientation, upstream of the NCTC8239 chromosomal *cpe* gene. The putative Shine-Dalgarno sequence for the F4969 *IS1469* sequences was determined to be identical to that of NCTC8239 (3). Upstream of *IS1469*, the plasmid *cpe* locus of

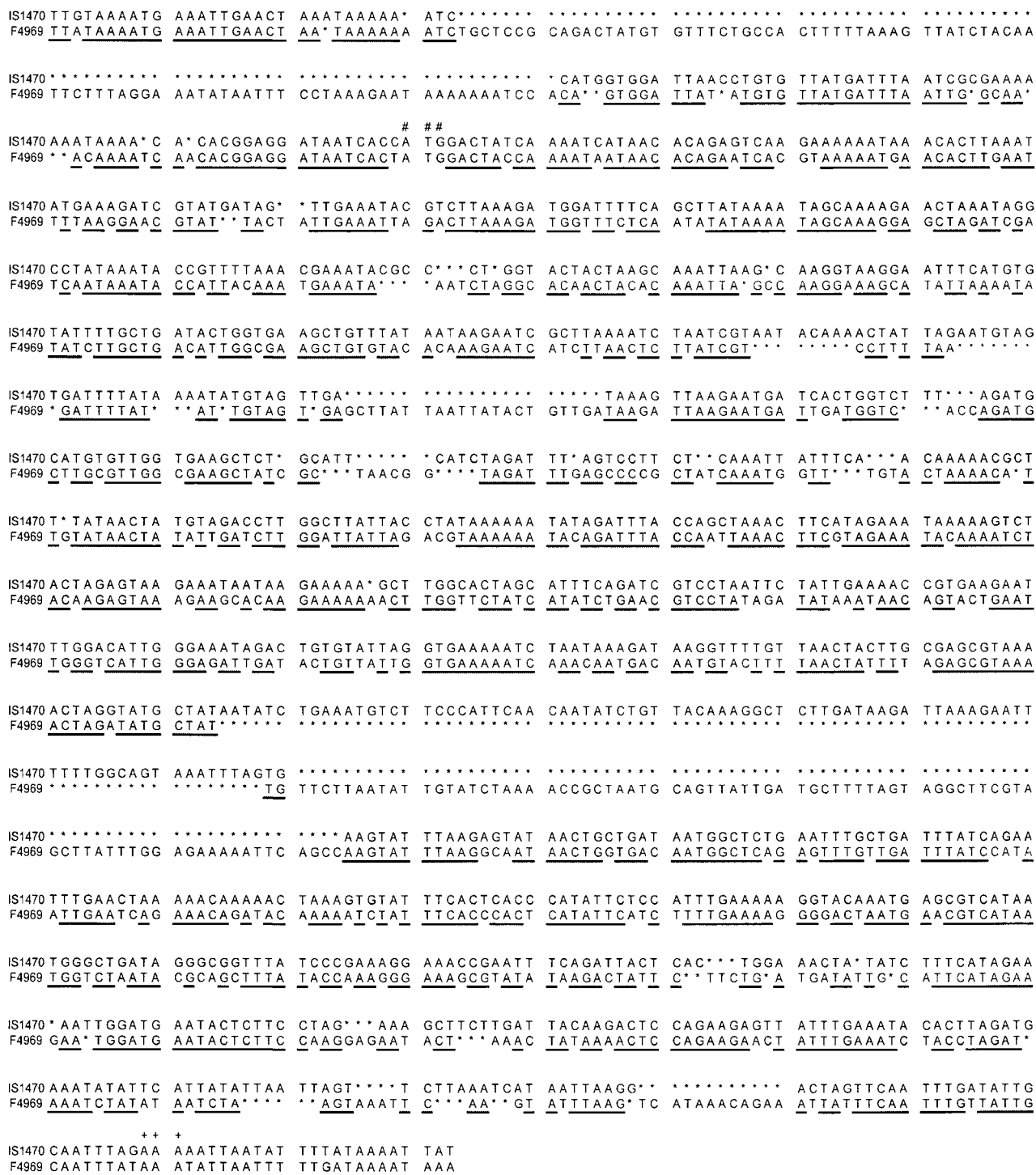


FIG. 2. Comparison of IS1470-like sequence in isolate F4969 and the downstream IS1470 sequence in the type A chromosomal-*cpe* food poisoning strain NCTC8239. #, putative start codon; +, putative stop codon. Underlined nucleotides are homologous between the IS1470 sequences downstream of the NCTC8239 *cpe* gene and the IS1470-like sequence of isolate F4969. *, missing nucleotide.

isolate F4969 was found to share homology, for another 200 bp, with the previously sequenced (3, 5) chromosomal *cpe* locus of strain NCTC8239. Beyond that conserved upstream region, significant sequence differences between these two *cpe*-

positive type A isolates were detected. Notably, the IS1470 sequences present directly upstream of the IS1469 element of NCTC8239 were not apparent in the plasmid *cpe* locus of isolate F4969. However, a partial ORF was identified at the

extreme 5' end of the *cpe*-containing *Xba*I fragment cloned from F4969 (Fig. 1). That partial ORF, absent from the chromosomal *cpe* locus of NCTC8239 (3, 5), is predicted to encode the C-terminal half of a protein sharing partial amino acid homology with the cytosine methyltransferase encoded by *Bacillus subtilis* phage SPBc2.

Finally, our sequencing analyses also identified six sequences near the plasmid *cpe* gene of strain F4969 that resemble consensus binding sequences for the Hpr (hyperprotease-producing) regulatory protein of *B. subtilis* (3, 5). Five of those putative Hpr-binding sequences were found to share complete homology with the previously detected putative Hpr-binding sequences present near the chromosomal *cpe* gene of NCTC8239 (3, 5). However, the upstream Hpr-binding sequence nearest the F4969 *cpe* gene has a single nucleotide replacement (AATAGTGTT is present instead of AATAGTATT).

PCR evaluation of the association between the *cpe* gene and IS1469 in other type A strains. To further investigate previous suggestions (5, 8) that IS1469 sequences are consistently associated with the *cpe* gene of *C. perfringens* type A isolates, comparative PCR analyses were performed with a varied collection of *cpe*-positive, type A isolates (Table 1). Those analyses, using one primer specific for *cpe* ORF sequences and a second primer specific for IS1469 sequences, amplified an ~1.7-kbp product using template DNA prepared from either F4969 or NCTC8239 (data not shown), which is fully consistent with expectations from KCpeFC5-1, KCpeFX28, and NCTC8239 sequencing results (3, 5; this study). A similar-sized PCR product was also amplified from all 16 other tested type A *cpe*-positive isolates (data not shown), regardless of their origin or *cpe* genotype (plasmid or chromosomal). However, no PCR product was amplified from the *cpe*-negative type A isolate MR2-2 (data not shown).

Detection of cytosine methyltransferase gene sequences (*dcm* sequences) in other *C. perfringens* strains. Following sequence analysis detection of putative *dcm* sequences near the F4969 *cpe* gene, two PCR studies were performed to evaluate whether similar *dcm* sequences might also be present in other *C. perfringens* type A isolates. As expected from KCpeFX28 sequencing results, a PCR assay using primers specific for internal F4969 *dcm* sequences amplified a single ~0.4-kbp band from F4969 template DNA. A similar product was also observed when template DNA from 11 of 12 other type A isolates carrying a plasmid *cpe* gene was used (Fig. 3A).

To further confirm the presence of *dcm* sequences in many *C. perfringens* type A isolates carrying a *cpe* plasmid, Southern blotting analysis was performed with a probe specific for F4969 *dcm* sequences. This probe hybridized with *Xba*I-digested DNA from all 10 type A isolates carrying a plasmid *cpe* gene (including isolate F5603, which had tested negative by our *dcm*-specific PCR assay). In all cases, the *dcm* probe hybridized to an *Xba*I fragment similar in size to a fragment that hybridized with a *cpe*-specific probe (Fig. 4A and B). DNA from type A isolate 452, which carries a *cpe* plasmid, also had a second band hybridizing with the *dcm*-specific probe; however, this band did not hybridize with the *cpe* probe (Fig. 4A and B).

In contrast to the apparently widespread presence of *dcm* sequences in type A isolates carrying a plasmid *cpe* gene, tem-

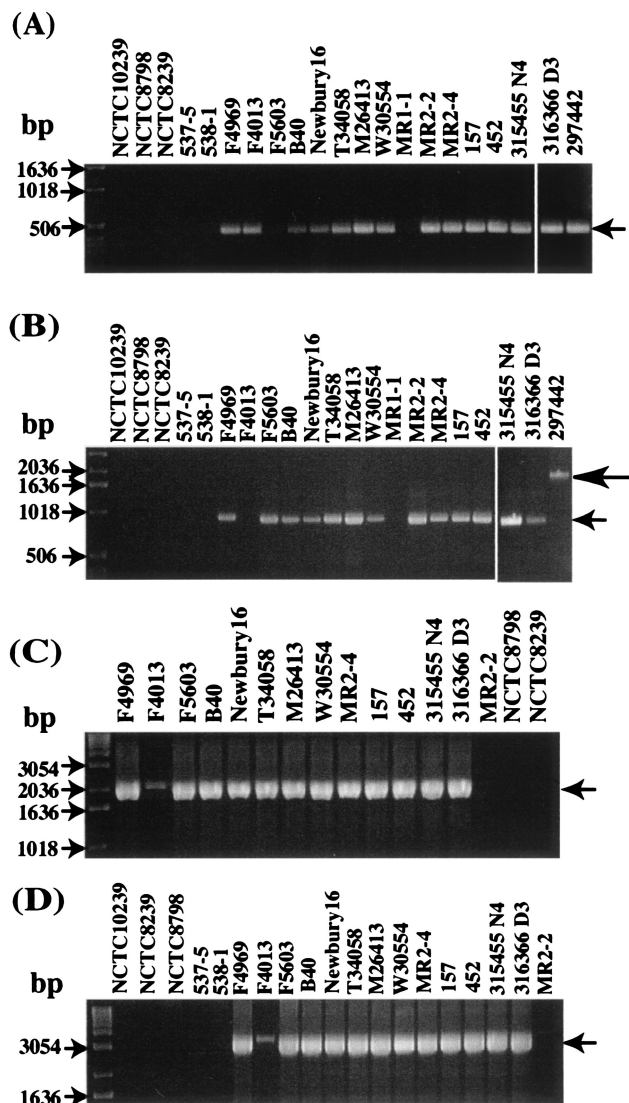


FIG. 3. PCR analyses of sequences upstream of the plasmid *cpe* gene of type A isolates. (A) Results of a *dcm*-specific PCR assay (expected ~0.4-kbp product marked by arrow) with primers internal to F4969 *dcm* sequences. (B) Results of a PCR assay performed with primers designed to amplify a product from the DNA region between *dcm* sequences and sequences ~400 bp downstream of them (based upon sequencing of the *cpe*-containing *Xba*I fragment of F4969). In this PCR assay, F4969 DNA yields an ~0.9-kbp PCR product (small arrow), which is consistent with sequencing results for the F4969 plasmid *cpe* locus; note that one *cpe*-negative type A isolate, 297442, gave an ~2.0-kbp product (large arrow). (C) PCR analysis of the association between *dcm* gene and IS1469 genes. An ~2.1-kbp product (arrow) was detected in all type A isolates carrying a plasmid *cpe* gene. (D) PCR analysis of the association between *dcm* sequences and the *cpe* gene, which is consistent with sequencing results for the plasmid *cpe* locus of F4969. An ~3.5-kbp product (arrow) was detected in all type A isolates carrying a plasmid *cpe* gene, which is consistent with sequencing results for the plasmid *cpe* locus of F4969. Numbers on the left indicate migration of molecular size markers.

plate DNA from five type A food poisoning strains carrying a chromosomal *cpe* gene failed to amplify a product in our *dcm*-specific PCR assay (Fig. 3A). Moreover, Southern blot analysis detected no hybridization of a *dcm* probe to *Xba*I-digested

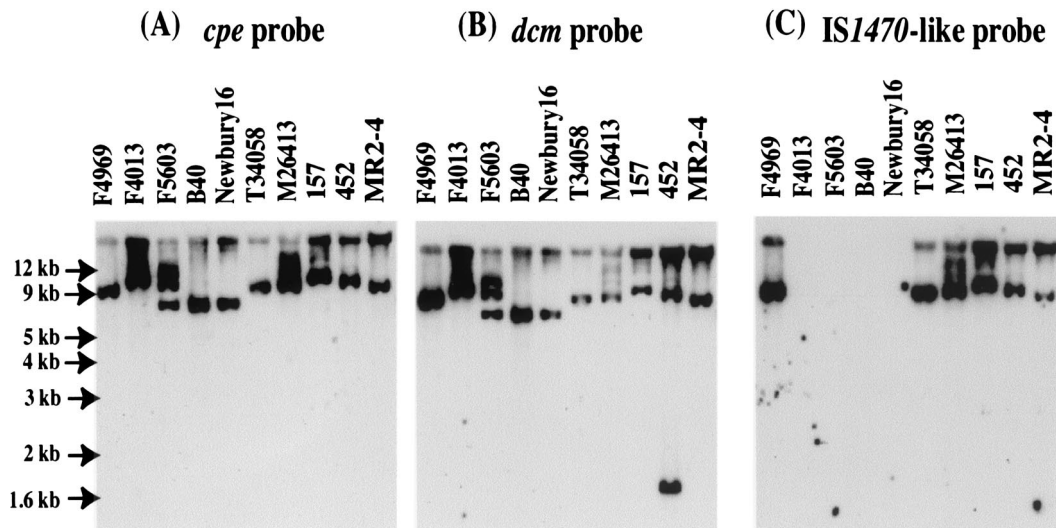


FIG. 4. Southern blot analyses of *cpe*-positive *C. perfringens* type A isolates. Results shown are for type A isolates carrying a *cpe* plasmid hybridized with a *cpe* probe, a *dcm* probe, or an IS1470-like probe. The uppermost bands in panel C lie in the gel wells. The F5603 lane shows hybridization of the probes to incompletely digested DNA. Numbers on the left indicate migration of molecular size markers.

DNA isolated from any of those five food poisoning isolates (Fig. 5). Finally, a PCR using *dcm*-specific primers and template DNA from three *cpe*-negative isolates (from both human and veterinary sources) amplified an ~0.4-kbp product from two of three template DNAs of those type A isolates (Fig. 3A).

A second PCR assay (using primers to F4969 *dcm* sequences

and F4969 sequences ~400 bp downstream of *dcm*) was then performed to further investigate the similarity of type A isolates carrying *dcm* sequences. As expected from KCpeFX28 sequencing results, this PCR amplified an ~0.9-kbp product by using F4969 template DNA (Fig. 3B). A similar product was also amplified with template DNA from 11 of 12 other type A isolates carrying a plasmid *cpe* (Fig. 3B). Interestingly, template DNA from plasmid *cpe* type A isolate F5603, which had tested negative with the *dcm*-specific PCR but positive by Southern blotting with a *dcm* probe, did amplify the expected 0.9-kbp product of our second PCR. In contrast, template DNA from type A plasmid *cpe* strain F4013, which tested positive by *dcm*-specific PCR, gave no PCR product with that second PCR.

Our second PCR using one primer to F4969 *dcm* sequences and a second primer to F4969 sequences downstream of *dcm* also failed to amplify any product from five type A food poisoning strains carrying a chromosomal *cpe* gene or from MR1-1, a *cpe*-negative type A isolate (Fig. 3B). DNA from another *cpe*-negative type A isolate (canine diarrhea isolate 297442) did amplify a larger (~2-kbp) product in this second PCR (Fig. 3B).

The possible presence of similar *dcm* sequences in other *C. perfringens* types was also investigated with representative (Table 1) type B, C, D, and E isolates. Template DNA prepared from isolates belonging to each *C. perfringens* type gave an ~0.4-kbp PCR product using primers specific for F4969 *dcm* sequences (Fig. 6A). Our second PCR, using primers to the *dcm* sequences and adjacent downstream sequences of F4969 plasmid DNA, amplified an ~2-kbp product with template DNA prepared from the representative type B, D, and E isolates (Fig. 6B). Both the ~0.4 and ~2-kbp PCR products from the type B, D, and E isolates also hybridized a *dcm*-specific probe (Fig. 6C and D).

The ~2-kbp PCR products amplified by using template DNA from the tested type B and E isolates as well as from

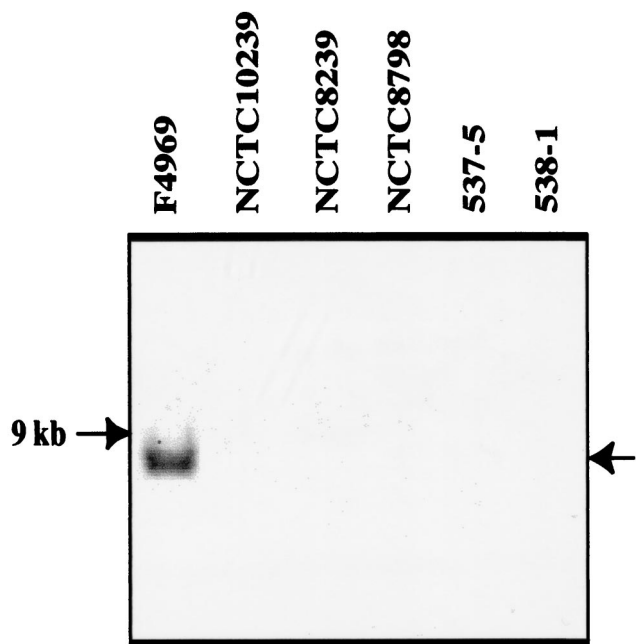


FIG. 5. Southern blotting analysis of *dcm* sequences in *C. perfringens* type A food poisoning isolates. Note that the probe, generated by PCR with primers to F4969 *dcm* sequences and F4969 sequences ~400 bp downstream of the *dcm* sequences, does not hybridize with DNA from any type A food poisoning isolates tested. However, this probe does react with DNA isolated from F4969.

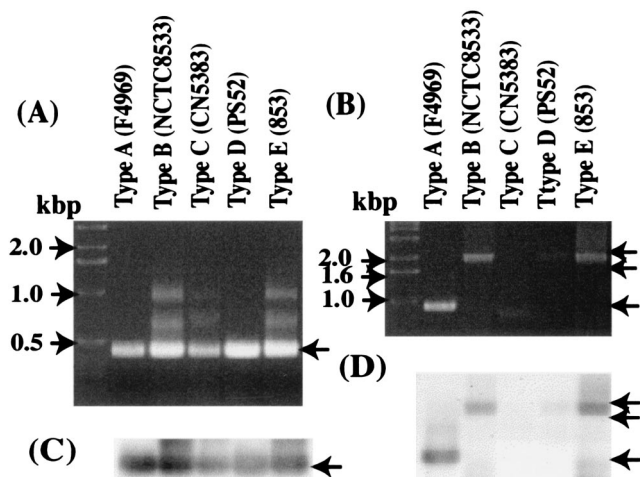


FIG. 6. PCR and Southern blotting analyses of putative cytosine methyltransferase gene sequences (*dcm* sequences) in other *C. perfringens* types. (A) PCR analyses with primers to internal *dcm* sequences; (C) Southern blotting results with a F4969 *dcm*-specific probe generated with the same primer pair and pKCpeFX28 template DNA. (A) This PCR assay generated an ~ 0.4 kbp product (arrow) in *C. perfringens* types A to E which is similar in size to the PCR product this primer pair generates with most type A strains (Fig. 3); those PCR products also hybridized to a *dcm*-specific probe (C). (B) PCR analysis using primers based on *dcm* sequences and sequences ~ 400 bp downstream from *dcm* sequences; (D) Southern blotting analysis with a *dcm*-specific probe. An ~ 0.9 -kbp product was detected in type A isolate F4969 (single arrow); otherwise, ~ 2.0 -kbp products were detected in type B, D, and E isolates (double arrow). Those products also hybridized with an F4969 *dcm*-specific probe. Numbers on the left indicate migration of molecular size markers.

cpe-negative type A isolate 297442 were directly sequenced to determine the basis for these unexpectedly larger (~ 2 - versus 0.9 -kbp) PCR products amplified with primers to *dcm* sequences and sequences downstream of *dcm* in F4969. The results confirmed the presence of similar *dcm* sequences in each of those isolates but also revealed the presence of an insertion containing an ~ 0.7 -kbp ORF that is predicted to encode a protein with partial homology to a hypothetical protein of *Streptococcus pneumoniae* bacteriophage MM1.

Comparative PCR analyses of DNA upstream of the plasmid *cpe* gene in type A isolates. The relationship between *dcm* sequences and IS1469 in type A isolates was investigated with a PCR using one primer complementary to F4969 *dcm* sequences and a second primer specific for the IS1469 sequences of F4969 (Fig. 3C). Consistent with our KCpeFX28 sequencing results, this PCR amplified a product of ~ 2.1 kbp using F4969 template DNA. A similar product was also amplified from all 12 other type A isolates carrying a plasmid *cpe* gene, although the amplification was weaker with F4013 template DNA. In contrast, two type A chromosomal-*cpe* isolates and one type A *cpe*-negative strain failed to amplify any PCR product in this reaction.

A second PCR was then performed using one primer complementary to *dcm* sequences and a second primer specific for *cpe* sequences (Fig. 3D). That PCR amplified a clear ~ 3.5 -kbp band using F4969 template DNA, consistent with KCpeFX28 sequencing results. A similar PCR product was also amplified

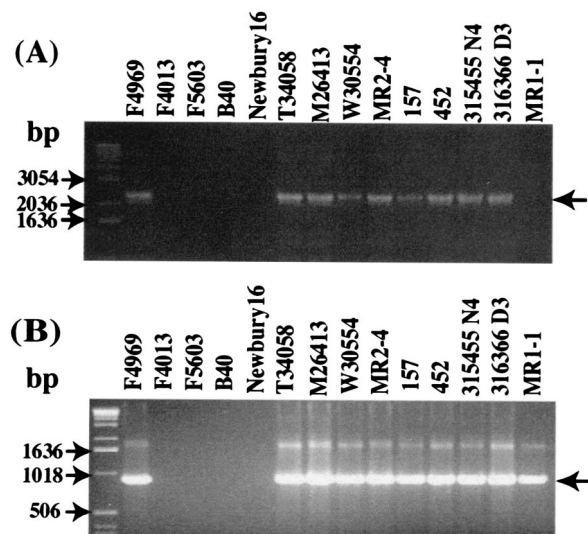


FIG. 7. PCR analyses of the region between the *cpe* gene and downstream IS1470-like sequences. (A) PCR product amplified from specified type A isolates using primers for *cpe* gene sequences and sequences present in IS1470-like sequences of F4969, which generate an ~ 2.2 -kbp product (arrow at right) with DNA from F4969. Numbers on the left indicate migration of molecular size markers. (B) PCR products amplified from specified type A isolates using primers specific for internal IS1470-like sequences. This reaction generated the expected 0.9 -kbp product from F4969 DNA (arrow). Numbers on the left indicate migration of molecular size markers.

with template DNA isolated from all 12 other type A isolates carrying a plasmid *cpe* gene, although the amplification was again weaker with template DNA from isolate F4013. However, five type A chromosomal-*cpe* strains and one type A *cpe*-negative strain failed to amplify any product in this PCR.

Comparative PCR and Southern blot analyses of the association between *cpe* and downstream IS1470-like sequences in *C. perfringens* type A isolates carrying a *cpe* plasmid. To investigate possible similarity in the region downstream of the plasmid *cpe* gene among type A isolates, a PCR was performed with a primer pair specific for *cpe* sequences and the IS1470-like sequence present in F4969 (Fig. 7A). As expected from KCpeFX28 sequencing results, this PCR amplified a product of ~ 2.2 kbp using F4969 template DNA. A similar product was also amplified with template DNA prepared from 8 of 12 other type A isolates carrying a plasmid *cpe* gene. By comparison, a chromosomal-*cpe* food poisoning isolate (not shown) and a type A *cpe*-negative isolate (Fig. 7A) both failed to amplify any product in this PCR.

To further investigate the association between IS1470-like sequences and the *cpe* gene in type A isolates carrying a *cpe* plasmid, Southern blotting analyses were performed with a probe specific for the IS1470-like sequences of F4969. Those experiments (Fig. 4C) detected hybridization of the IS1470-like probe to *Xba*I-digested DNA isolated from five of nine type A isolates carrying a plasmid *cpe* gene. For each of those five isolates, a similar-sized *Xba*I fragment always hybridized to both the *cpe* and IS1470-like probes. The four type A isolates (F4013, F5603, B40, and NB16) carrying a plasmid *cpe* gene whose DNA failed to hybridize with the IS1470-like probe in

the Southern blots (Fig. 4) were also unable to amplify a *cpe-IS1470*-like PCR product (Fig. 7A).

As a final assessment of the presence of *IS1470*-like sequences in type A isolates carrying a plasmid *cpe* gene, a second PCR was performed with primers to internal *IS1470*-like sequences (Fig. 7B). With those internal *IS1470*-like primers, template DNA from F4969 amplified the expected ~0.9-kbp PCR product. The eight other type A plasmid *cpe* isolates whose DNA had amplified (Fig. 7A) a PCR product with *cpe* and *IS1470*-like primers also gave a similar ~0.9-kbp PCR product with the internal *IS1470*-like primers. However, those same primers failed to support amplification of any PCR product (Fig. 7B) with template DNA from F4013, F5603, B40, or NB16, which is consistent with the fact that DNA from those four isolates failed to either hybridize an *IS1470*-like probe or amplify a *cpe-IS1470*-like PCR product (Fig. 4 and 7).

PCR detection of *IS1151* sequences. Since previous studies had detected *IS1151* sequences near the plasmid *cpe* gene in two of three type A isolates (8), a PCR assay was performed with *IS1151*-specific primers and template DNAs prepared from our collection of type A isolates carrying a plasmid *cpe* gene. As shown in Fig. 8A, the internal *IS1151* primers amplified a PCR product of ~0.6 kbp using template DNA from type E isolate 853, consistent with previous sequencing results (1). A similar-sized PCR product was also detected when the same reaction was performed with template DNA prepared from type A plasmid *cpe* isolate F4013 (Fig. 8A). However, this PCR failed to amplify any product using template DNA from F4969 (Fig. 8A), the 11 other type A strains carrying a plasmid *cpe* gene that had also been tested in analyses whose results are shown in Fig. 7 (data not shown), or one type A *cpe*-negative strain (data not shown).

To evaluate the proximity of the *cpe* gene to the putative *IS1151* sequences of isolate F4013 and to further confirm the absence of *IS1151* sequences in the other 12 type A isolates carrying a plasmid *cpe* gene examined by the internal *IS1151* PCR, a second PCR was performed with one primer specific for *cpe* sequences and a second primer specific to *IS1151* sequences. As shown in Fig. 8B, these primers amplified a PCR product using DNA from a single type A isolate carrying a *cpe* plasmid, i.e., F4013. No product was amplified from F4969 (Fig. 8B) or from the other 12 type A isolates also testing negative with the internal *IS1151* PCR assay (data not shown).

Sequencing of the ~1.1-kb *cpe-IS1151* PCR product amplified from isolate F4013 DNA confirmed the presence of sequences resembling *IS1151* ~0.3 kbp downstream of the F4013 plasmid *cpe* gene (Fig. 1). The amplified F4013 sequences were determined to be ~90% homologous to *IS1151* but appear to be defective, as they contain several nonsense mutations. In addition, this sequencing analysis revealed that the region of F4013 DNA containing *IS1151*-like sequences also has an overlapping ORF (in a different reading frame from *IS1151*) sharing partial amino acid homology with transposase 11.

To further evaluate the presence of sequences with homology to *IS1151* in the plasmid *cpe* locus of type A isolates, a Southern blot experiment was performed with a probe specific for internal *IS1151*-like sequences of isolate F4013. These analyses (Fig. 8C) demonstrated hybridization of the *IS1151*-

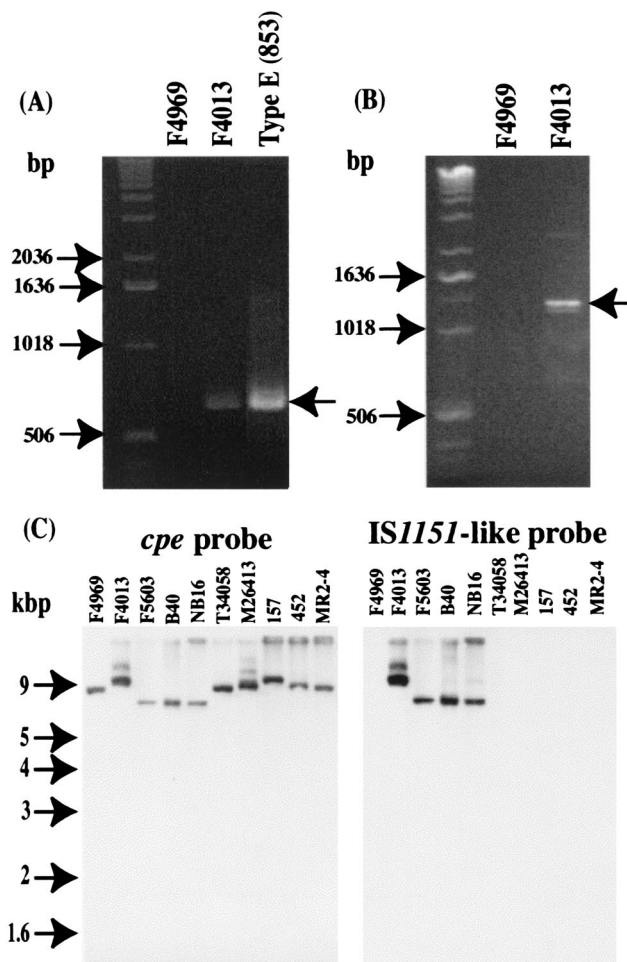


FIG. 8. PCR detection of *IS1151* sequences in *C. perfringens* type A isolates. (A) PCR results obtained with primers specific for *IS1151* sequences which amplified (arrow at right) the expected ~0.6-kbp product with total DNA from the previously sequenced *IS1151* of type E isolate 853 (1). Numbers on the left indicate migration of molecular weight markers. (B) PCR results obtained with a primer specific for *cpe* sequences and a reverse primer specific for *IS1151* sequences. Sequencing of the ~1.2-kbp product obtained with F4013 DNA confirmed the inclusion of sequences spanning from *cpe* to *IS1151*. (C) Southern blot results obtained by hybridizing *Xba*I-digested DNA from type A isolates carrying a *cpe* plasmid with an internal *IS1151*-like probe.

like probe to a single *Xba*I DNA fragment from the type A plasmid *cpe* isolates F4013, F5603, B40, and NB16. For each of these isolates, a *cpe* probe also apparently hybridized to the *Xba*I fragment hybridizing to the *IS1151*-like probe. Coupling this observation with the apparent presence (Fig. 3) in these isolates of an F4969-like DNA region upstream of *cpe* (which should include the *Xba*I site of *dcm* sequences), the *IS1151*-like sequences of F5603, B40, and NB16 (like isolate F4013) appear to be located downstream of their *cpe* gene. No hybridization of the *IS1151*-like probe was observed with DNA prepared from any other type A isolates carrying a *cpe* plasmid (Fig. 8C), which all carry *IS1470*-like sequences downstream of their *cpe* gene (Fig. 7).

DISCUSSION

Recent studies (2, 3, 5–8, 23) established that most (or all) *C. perfringens* type A food poisoning isolates carry their enterotoxin gene (*cpe*) on the chromosome. Sequencing studies suggested that the chromosomal *cpe* gene of type A food poisoning isolate NCTC8239 (2, 3, 5, 10) may be present on Tn5565, a putative 6.3-kbp transposon with terminal *IS1470* elements. Tn5565 has apparently inserted onto the *C. perfringens* chromosome between the purine permease (*uapC*) and quinolinate phosphorylribosyltransferase (*nadC*) genes (Fig. 1). Southern blot results suggest that other type A food poisoning strains have a similarly arranged chromosomal *cpe* locus (5, 8).

In contrast, most (if not all) enterotoxigenic *C. perfringens* type A isolates from non-food-borne human GI diseases or veterinary sources carry their *cpe* gene on a large plasmid. Prior to the present study, the organization of the plasmid *cpe* locus in type A isolates had not yet been thoroughly investigated, although an initial Southern blot study (8) suggested that the plasmid *cpe* locus of type A isolates substantially differs from the well-studied chromosomal *cpe* locus of NCTC8239. That study did not detect *IS1470* sequences in the plasmid *cpe* locus of three tested type A isolates but instead found *IS1151* sequences downstream of the *cpe* gene in two of those three isolates. Since those initial findings, the *IS1151*-containing *cpe* locus of F3686 (the only previously examined type A plasmid *cpe* isolate associated with human disease) has been considered representative of type A isolates carrying a *cpe* plasmid.

However, the apparent presence of *IS1151* sequences in only two of the three initially examined isolates indicated that some variation must exist between the plasmid *cpe* loci of different type A isolates. To better evaluate the organization and diversity of the plasmid *cpe* locus in type A isolates, we first sequenced the plasmid *cpe* locus of F4969, a type A isolate from non-food-borne human GI disease used previously in both pathogenesis and genetic studies (4, 20). The arrangement determined for the plasmid *cpe* locus of F4969 (Fig. 1) was then compared, by PCR and Southern blotting, against the organization of the *cpe* locus in other type A strains carrying a *cpe* plasmid.

The results of this study are important for several reasons. First, the availability of sequence data for the F4969 plasmid *cpe* locus permits direct, detailed comparison of the plasmid *cpe* locus of a type A isolate against the previously sequenced chromosomal *cpe* locus of type A food poisoning strain NCTC8239 (3, 5, 10). Such comparisons indicate that the *cpe* loci of F4969 and NCTC8239 share nearly complete homology for ~4 kbp. This conserved region extends ~1.2 kbp downstream from the (~1-kbp) *cpe* gene. Immediately beyond this common downstream region (Fig. 1), NCTC8239 carries *IS1470* sequences, while F4969 has (in nearly the same position) *IS1470*-like sequences that are defective and present in the orientation opposite that of the NCTC8239 *IS1470* sequences. Identification of *IS1470*-like sequences downstream of the plasmid *cpe* gene of F4969 contradicts previous studies that failed to find sequences with homology to *IS1470* associated with the plasmid *cpe* locus of type A isolates (8).

With the exception of a 45-bp insert directly upstream of the F4969 *cpe* gene, F4969 and NCTC8239 were also found to

share considerable homology for ~1.8 kbp upstream of their *cpe* genes (Fig. 1). This result is consistent with previous proposals (3, 5, 8) that *IS1469* sequences are located immediately upstream of the plasmid *cpe* gene in most (or all) type A isolates (see further discussion below). Approximately 250 bp beyond those shared *IS1469* sequences, significant sequence divergence was detected between the *cpe* locus of F4969 and that of NCTC8239. In that divergent upstream region, F4969 lacks the *IS1470* sequences present immediately upstream of the NCTC8239 *IS1469* element (Fig. 1). Instead, sequences including the 3' half of a putative cytosine methyltransferase gene (*dcm*) were identified ~1.2 kbp upstream of the *IS1469* element in isolate F4969 (Fig. 1).

This study also offers an improved assessment of plasmid *cpe* locus diversity among type A isolates. Using a collection of 13 type A isolates carrying a plasmid *cpe* gene from varied geographic and host or disease origins, it was determined that (as for all chromosomal-*cpe* isolates thus far examined [3, 5, 8; this study]) *IS1469* sequences are present immediately upstream of the plasmid *cpe* gene in type A isolates. This finding further supports the already-mentioned proposals (3, 5, 8) of a consistent association between the *cpe* gene and *IS1469*. Additionally, *dcm* sequences (which are absent from chromosomal-*cpe* isolates) were localized to the same position upstream of the *cpe* gene in all 13 tested type A isolates carrying a plasmid *cpe* gene. Thus, results of this study strongly suggest that, for at least 3.5 kbp, sequences upstream of the *cpe* gene are conserved in most (or all) type A isolates carrying a *cpe* plasmid.

Our results also indicate that *dcm* sequences (when present) are not restricted to type A isolates carrying a *cpe* plasmid. Two of three *cpe*-negative type A strains from normal human and veterinary sources were also determined to carry *dcm* sequences, as were representative type B, C, D, and E isolates. Interestingly, a primer pair specific for *dcm* sequences and sequences located 400 bp downstream of *dcm* in strain F4969 DNA was able to amplify a larger (relative to the product generated with F4969 DNA) PCR product using template DNA from type A *cpe*-negative strain 297442 or representative type B, D, and E isolates, which all carry plasmids (21). Sequencing demonstrated that this larger PCR product has an insert containing an ORF with partial homology to a protein encoded by *S. pneumoniae* bacteriophage MM1. Since the *dcm* sequences of the F4969 *cpe* plasmid (and, by inference, other *dcm*-positive *C. perfringens* isolates) also share partial homology with a *dcm* gene found in *B. subtilis* phage SPBc2, future studies might explore whether some *C. perfringens* plasmids have a phage origin or have recombined with phage DNA. Further study is also warranted to examine whether *dcm* sequences are present on the virulence plasmids encoding beta, epsilon, and iota toxins in type B to E isolates. It also should be determined whether the detected *dcm* sequences actually express a functional cytosine methylase and, if so, whether that methylase plays any role in *C. perfringens* gene regulation.

The diversity of the DNA region downstream of the plasmid *cpe* gene of type A isolates was also addressed by the present study. When 13 randomly chosen type A plasmid *cpe* isolates from varied geographic and host or disease sources were surveyed, only 4 (~30%) were found to carry *IS1151*-like sequences (which are defective) downstream of their *cpe* gene. Thus, contrary to initial indications (8), it now appears that

organization of the plasmid *cpe* locus found in IS1151-like carrying type A isolates such as F3686 and F4013 is less representative than the IS1470-like containing plasmid *cpe* locus found in isolates such as F4969. The inability of our PCR assays to amplify a product from F5603, B40, or NB16 template DNA using primers specific for the IS1151-like sequences of F4013 suggests that diversity exists among the IS1151-like sequences of different type A isolates carrying a *cpe* plasmid. Finally, the failure of the present study to find more than two distinctly different types of organization in the plasmid *cpe* locus of type A isolates among 13 isolates suggests that that locus has only limited diversity.

The ever-increasing evidence for a close association between IS1469 sequences and the *cpe* gene in most (or all) type A isolates (3, 5, 8; this study) supports the possibility of chromosomal and plasmid *cpe* genes sharing a common origin. Since the NCTC8239 *cpe* gene is apparently (2, 5) located on Tn5565, a putative transposon with terminal IS1470 sequences that reportedly forms several circular intermediates (including an IS1469-*cpe* species without any IS1470 sequences), previous proposals (2, 5) that the plasmid *cpe* locus of type A isolates originated from integration of an IS1469-*cpe* genetic element into one or more *C. perfringens* plasmids remain viable. If so, the IS1470-like and IS1151-like sequences now present downstream of the plasmid *cpe* gene in type A isolates (8; this study) could reflect insertion events occurring after integration of the IS1469-*cpe* element onto plasmids.

Alternatively, discovery of IS1470-like sequences downstream of the plasmid *cpe* gene in F4969 and many other type A isolates opens the possibility of independent origins for the F4969-like and F4013-like plasmid *cpe* loci. A possible origin of the F4013-like plasmid *cpe* locus is discussed above, i.e., integration of an IS1469-*cpe* genetic element followed by a subsequent integration of an IS1151 element downstream of the *cpe* gene. In contrast, the F4969-like plasmid *cpe* locus could reflect integration of another *cpe*-containing genetic element (consisting of IS1469, *cpe*, and a single IS1470 sequence) onto a different *C. perfringens* plasmid. This hypothesis receives some support from previous PCR studies (2) purportedly identifying an IS1469-*cpe*-IS1470 circular intermediate in NCTC8239 DNA (2). However, for the F4969-like plasmid *cpe* locus to have this independent origin, some DNA rearrangements must have occurred during transposition to account for the reversed orientation of the IS1470-like sequences present in the F4969-like plasmid *cpe* locus. Finally, if IS1469-*cpe* and IS1469-*cpe*-IS1470 genetic elements have independently inserted onto different *C. perfringens* plasmids, the conserved nature of the upstream region of the plasmid *cpe* locus in most or all *C. perfringens* type A isolates, and the presence of *dcm* sequences in some *cpe*-negative isolates carrying plasmids, could suggest that plasmid DNA sequences downstream of *dcm* represent an insertional hot spot.

Regardless of how many different *cpe*-carrying genetic elements have integrated into *C. perfringens* plasmids, our results show that functional upstream and downstream IS1470 sequences are missing from the plasmid *cpe* locus of most (or all) type A isolates. If IS1470 insertion sequences are necessary for transposing the *cpe* gene of NCTC8239, as proposed elsewhere (2, 5), it is possible that the plasmid *cpe* gene of most type A isolates can no longer be mobilized from resident plasmids.

This putative loss of function (i.e., mobilization of the *cpe* gene) could explain why large numbers of mutations, including nonsense mutations, are now present in the IS1470-like sequences of type A isolates carrying a plasmid with the F4969 type of plasmid *cpe* locus.

Finally, it is notable that an F4969-like plasmid *cpe* locus is also present in isolate 452, which was previously shown to lack a clonal relationship with F4969 (6). The presence of a similar plasmid *cpe* locus in two unrelated type A isolates, from very different origins, is consistent with the possibility of these two type A isolates now sharing the same *cpe* plasmid. If true, this situation could reflect horizontal spread of a common plasmid carrying an F4969-like *cpe* locus to many other *C. perfringens* type A isolates, converting the recipients to enteric virulence. This hypothesis is consistent with recent studies demonstrating that the *cpe* plasmid of type A strain F4969 can transfer via conjugation (4). Studies are now under way to further assess the similarities between the *cpe* plasmids in different *C. perfringens* type A isolates and to evaluate if plasmids with the F4013 type of *cpe* locus organization can also transfer via conjugation.

ACKNOWLEDGMENTS

This research was supported by Public Health Service grant AI19844 from the National Institute of Allergy and Infectious Diseases and by grant 2001-02517 from the Ensuring Food Safety Research Program of the U.S. Department of Agriculture.

We thank Shauna Sparks for technical assistance, Robert Carman for providing canine diarrhea isolates 315455N4, 316366D3, and 297442, and Julian Rood (Monash University, Melbourne, Australia) for helpful discussions.

REFERENCES

1. Billington, S. J., E. U. Wieckowski, M. R. Sarker, D. Bueschel, J. G. Songer, and B. A. McClane. 1998. *Clostridium perfringens* type E animal enteritis isolates with highly conserved, silent enterotoxin sequences. *Infect. Immun.* **66**:4531-4536.
2. Brynestad, S., and P. E. Granum. 1999. Evidence that Tn5565, which includes the enterotoxin gene in *Clostridium perfringens*, can have a circular form which may be a transposition intermediate. *FEMS Microbiol. Lett.* **170**:281-286.
3. Brynestad, S., L. A. Iwanejko, G. S. A. B. Stewart, and P. E. Granum. 1994. A complex array of Hpr consensus DNA recognition sequences proximal to the enterotoxin gene in *Clostridium perfringens* type A. *Microbiology* **140**:97-104.
4. Brynestad, S., M. R. Sarker, B. A. McClane, P. E. Granum, and J. I. Rood. 2001. Enterotoxin plasmid from *Clostridium perfringens* is conjugative. *Infect. Immun.* **69**:3483-3487.
5. Brynestad, S., B. Synstad, and P. E. Granum. 1997. The *Clostridium perfringens* enterotoxin gene is on a transposable element in type A human food poisoning strains. *Microbiology* **143**:2109-2115.
6. Collie, R. E., J. F. Kokai-Kun, and B. A. McClane. 1998. Phenotypic characterization of enterotoxigenic *Clostridium perfringens* isolates from non-foodborne human gastrointestinal diseases. *Anaerobe* **4**:69-79.
7. Collie, R. E., and B. A. McClane. 1998. Evidence that the enterotoxin gene can be episomal in *Clostridium perfringens* isolates associated with non-foodborne human gastrointestinal diseases. *J. Clin. Microbiol.* **36**:30-36.
8. Cornillot, E., B. Saint-Joanis, G. Daube, S. Katayama, P. E. Granum, B. Carnard, and S. T. Cole. 1995. The enterotoxin gene (*cpe*) of *Clostridium perfringens* can be chromosomal or plasmid-borne. *Mol. Microbiol.* **15**:639-647.
9. Czeczulin, J. R., R. E. Collie, and B. A. McClane. 1996. Regulated expression of *Clostridium perfringens* enterotoxin in naturally *cpe*-negative type A, B, and C isolates of *C. perfringens*. *Infect. Immun.* **64**:3301-3309.
10. Czeczulin, J. R., P. C. Hanna, and B. A. McClane. 1993. Cloning, nucleotide sequencing, and expression of the *Clostridium perfringens* enterotoxin gene in *Escherichia coli*. *Infect. Immun.* **61**:3429-3439.
11. Daube, G., P. Simon, and A. Kaeckenbeek. 1993. IS1151, an IS-like element of *Clostridium perfringens*. *Nucleic Acids Res.* **21**:352.
12. Daube, G., P. Simon, B. Limbourg, C. Manteca, J. Maimil, and A. Kaeckenbeek. 1996. Hybridization of 2,659 *Clostridium perfringens* isolates with gene

- probes for seven toxins (α , β , ϵ , ι , θ , μ and enterotoxin) and for sialidase. *Am. J. Vet. Res.* **57**:496–501.
13. **Kokai-Kun, J. F., J. G. Songer, J. R. Czczulin, F. Chen, and B. A. McClane.** 1994. Comparison of Western immunoblots and gene detection assays for identification of potentially enterotoxigenic isolates of *Clostridium perfringens*. *J. Clin. Microbiol.* **32**:2533–2539.
 14. **McClane, B. A.** 2001. *Clostridium perfringens*, p. 351–372. In M. P. Doyle, L. R. Beuchat, and T. J. Montville (ed.), *Food microbiology: fundamentals and frontiers*, 2nd ed. ASM Press, Washington, D.C.
 15. **McClane, B. A., D. M. Lyerly, J. S. Moncrief, and T. D. Wilkins.** 2000. Enterotoxigenic clostridia: *Clostridium perfringens* type A and *Clostridium difficile*, p. 551–562. In V. A. Fischetti, R. P. Novick, J. J. Ferretti, D. A. Portnoy, and J. I. Rood (ed.), *Gram-positive pathogens*. ASM Press, Washington, D.C.
 16. **McClane, B. A., and J. I. Rood.** 2001. Clostridial toxins involved in human enteric and histotoxic infections, p. 169–209. In H. Bahl and P. Dürre (ed.), *Clostridia: biotechnology and medical applications*. Wiley-VCH, Weinheim, Germany.
 17. **Melville, S. B., R. Labbe, and A. L. Sonenshein.** 1994. Expression from the *Clostridium perfringens* *cpe* promoter in *C. perfringens* and *Bacillus subtilis*. *Infect. Immun.* **62**:5550–5558.
 18. **Roberts, I., W. M. Holmes, and P. B. Hylemon.** 1986. Modified plasmid isolation method for *Clostridium perfringens* and *Clostridium absonum*. *Appl. Environ. Microbiol.* **52**:197–199.
 19. **Sambrook, J. E., E. F. Fritsch, and T. Maniatis.** 1989. *Molecular cloning: a laboratory manual*, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
 20. **Sarker, M. R., R. J. Carman, and B. A. McClane.** 1999. Inactivation of the gene (*cpe*) encoding *Clostridium perfringens* enterotoxin eliminates the ability of two *cpe*-positive *C. perfringens* type A human gastrointestinal disease isolates to affect rabbit ileal loops. *Mol. Microbiol.* **33**:946–958.
 21. **Songer, J. G.** 1996. Clostridial enteric diseases of domestic animals. *Clin. Microbiol. Rev.* **9**:216–234.
 22. **Songer, J. G., and R. M. Meer.** 1996. Genotyping of *Clostridium perfringens* by polymerase chain reaction is a useful adjunct to diagnosis of clostridial enteric disease in animals. *Anaerobe* **2**:197–203.
 23. **Sparks, S. G., R. J. Carman, M. R. Sarker, and B. A. McClane.** 2001. Genotyping of enterotoxigenic *Clostridium perfringens* isolates associated with gastrointestinal disease in North America. *J. Clin. Microbiol.* **39**:883–888.
 24. **Zhao, Y., and S. B. Melville.** 1998. Identification and characterization of sporulation-dependent promoters upstream of the enterotoxin gene (*cpe*) of *Clostridium perfringens*. *J. Bacteriol.* **180**:136–142.

Editor: D. L. Burns