Molecular Characterization of *Clostridium perfringens* Isolates from Humans with Sporadic Diarrhea: Evidence for Transcriptional Regulation of the Beta2-Toxin-Encoding Gene

Ben Harrison,1 Deepa Raju,1 Helen S. Garmory,2 Moira M. Brett,3 Richard W. Titball,2 and Mahfuzur R. Sarker1*

1Department of Microbiology, Oregon State University, Corvallis, Oregon 97331; 2Department of Biomedical Sciences, Defence Science and Technology Laboratory, Porton Down, Salisbury, Wilshire SP4 0JQ, United Kingdom; and 3Health Protection Agency and Reference Microbiology Division, London NW9 5HT, United Kingdom

Received 18 May 2005/Accepted 6 September 2005

*Clostridium perfringens* type A food poisoning is caused by *C. perfringens* isolates carrying a chromosomal enterotoxin gene (*cpe*), while non-food-borne gastrointestinal (GI) diseases, such as antibiotic-associated diarrhea (AAD) and sporadic diarrhea (SD), are caused by *C. perfringens* plasmid *cpe* isolates. A recent study reported the association of beta2 toxin (CPB2) with human GI diseases, and particularly AAD/SD, by demonstrating that a large percentage of AAD/SD isolates, in contrast to a small percentage of food poisoning isolates, carry the beta2-toxin gene (*cpb2*). This putative relationship was further tested in the current study by characterizing 14 *cpe*+ *C. perfringens* fecal isolates associated with recent cases of human SD in England (referred to hereafter as SD isolates). These SD isolates were all classified as *cpe*+ type A, and 12 of the 14 *cpe*+ isolates carry their *cpe* gene on the plasmid and 2 carry it on the chromosome. Interestingly, *cpb2* is present in only 12 plasmid *cpe* isolates; 11 isolates carry *cpe* and *cpb2* on different plasmids, but *cpe* and *cpb2* are located on the same plasmid in one isolate. *C. perfringens* enterotoxin is produced by all 14 *cpe*+ SD isolates. However, only 10 of the 12 *cpe*+/*cpb2*+ SD isolates produced CPB2, with significant variation in amounts. The levels of *cpb2* mRNA in low- to high-CPB2-producing SD isolates differed to such an extent (30-fold) that this difference could be considered a major cause of the differential level of CPB2 production in vitro by SD isolates. Furthermore, no silent or atypical *cpb2* was found in a CPB2 Western blot-negative isolate, 5422/94, suggesting that the lack of CPB2 production in 5422/94 was due to low expression of *cpb2* mRNA. This received support from our observation that the recombinant plasmid carrying 5422/94 *cpb2*, which overexpressed *cpb2* mRNA, restored CPB2 production in F4969 (a *cpb2*-negative isolate). Collectively, our present results suggest that CPB2 merits further study as an accessory toxin in *C. perfringens*-associated SD.

*Clostridium perfringens* is an important cause of histotoxic and gastrointestinal (GI) diseases in both humans and animals (19, 24). The virulence of this bacterium largely results from its ability to produce at least 15 different toxins (12, 20). *C. perfringens* isolates are commonly classified (20) into one of five types, A to E, based upon their ability to produce the four “major lethal toxins” (i.e., alpha, beta, epsilon, and iota toxins). The major lethal toxins, however, are not the only biomedically important toxins; some *C. perfringens* isolates (mostly belonging to type A) produce *C. perfringens* enterotoxin (CPE) (19), and many of both types A and C produce β2 toxin (CPB2) (5, 10, 12, 29).

CPE-positive *C. perfringens* type A strains are very important human GI pathogens, causing *C. perfringens* type A food poisoning and non-food-borne human GI diseases such as antibiotic-associated diarrhea (AAD) and sporadic diarrhea (SD) (19). Several studies (7, 8, 25) have shown that *C. perfringens* type A food poisoning isolates carry the gene (cpe) encoding CPE on the chromosome, while *cpe* is located on a plasmid in AAD/SD isolates. A recent study (10) demonstrated that some AAD/SD isolates carry *cpe* and the gene (*cpb2*) encoding CPB2 on the same plasmid when IS1151 sequences are present downstream of *cpe*, but *cpe* and *cpb2* are located on different plasmids in AAD/SD isolates where IS1470-like sequences are present downstream of *cpe*. Substantial experimental and epidemiological evidence (19, 22) now indicates that CPE plays a major role in the development of GI symptoms in cases of *C. perfringens*-associated food-borne and non-food-borne diseases. However, the possibility that other toxins also contribute to the development of these GI symptoms cannot be excluded.

Several epidemiological studies suggested that *cpb2*-positive *C. perfringens* isolates are highly associated with enteric diseases in domestic animals, particularly with enteritis in piglets (11, 12, 16, 29), typhlocolitis in horses (1, 13, 28), diarrhea in dogs (26), ulcerative enteritis in an African elephant (2), and enterotoxemia in calves (18). Although *cpb2* was initially identified in *C. perfringens* type C isolates, a recent study (5) demonstrated the presence of *cpb2* sequences in all types of *C. perfringens*. Although the deduced amino acid sequence of CPB2 is highly conserved in type A and C strains of *C. perfringens* isolated from diarrheic pigs (12, 29), the CPB2 sequence variation was observed in type A human AAD/SD isolates (10) and in isolates from nonporcine animals (15).

Recently Fisher et al. (10) reported, for the first time, the association of CPB2 with human GI diseases, particularly AAD/SD, by demonstrating that (i) a large percentage of
human AAD/SD isolates, in contrast to a small percentage of food poisoning isolates, produce the CPB2 toxin and ii) purified CPB2 from human AAD/SD isolates is cytotoxic to CaCo-2 cells, a commonly used in vitro model for the human intestinal epithelium. To test further the association of CPB2 with human GI diseases, our current study has characterized 14 cpe- C. perfringens fecal isolates associated with recent cases of human SD in England (27). Results from the current study indicate that all surveyed C. perfringens plasmid cpe isolates carry cpb2 and that there is a positive correlation between cpb2 transcription levels and the amount of CPB2 produced by cpe-cpb2 C. perfringens human SD isolates.

**MATERIALS AND METHODS**

**Bacterial strains and growth conditions.** The C. perfringens SD isolates used in this study are listed and described in Table 1. C. perfringens isolates used as controls in this study included F4969, a cpe-cpb2, type A isolate carrying the cpe gene on a plasmid (7); FS603, a cpe-cpb2 type A isolate carrying cpe and cpb2 on the same plasmid (10); F4396, a cpe-cpb2, type A isolate carrying cpe and cpb2 on two different plasmids (10); NCTC8239, a cpe type A isolate carrying the cpe gene on the chromosome (7); ATCC 3624, a cpe-cpb2 type A isolate (7, 10); CW245, a cpb2-positive type C pig isolate (12); JGS1807, a cpe-cpb2 type A pig isolate (29); and 106527, a cpb2-negative type C pig isolate (12). A starter culture (6 ml) of each C. perfringens isolate was prepared by overnight growth at 37°C in fluid thioglycolate broth (FTG) (Difco) as described previously (9). For DNA and RNA isolation or culture supernatant protein preparation, an aliquot (0.2 ml) of each FTG culture was inoculated into 10 ml of TGY broth (3% Trypticase, 2% glucose, 1% yeast extract, 0.1% cysteine) (9), which was then incubated at 37°C for 5 h to 24 h without shaking. (Note that, because of moderate aerotolerance, C. perfringens can grow in liquid medium without anaerobic incubation.) For selecting C. perfringens transformants carrying pDR42, C. perfringens cultures were plated onto brain heart infusion agar plates containing erythromycin (50 μg/ml) and incubated at 37°C for 18 h in an anaerobic jar with BBL GasPak (Becton, Dickinson and Company).

**Multiplex PCR toxin genotyping of C. perfringens isolates.** Total C. perfringens DNA was isolated from the overnight TGY cultures by using a previously described protocol (9, 22). That isolated DNA was then subjected, as described previously (11, 21), to multiplex PCR diagnostic screening for detection of gene sequences encoding C. perfringens alpha toxin, beta toxin, beta2 toxin, epsilon toxin, iota toxin, and CPE. After multiplex PCR, the presence of amplified toxin gene sequences was then analyzed by subjecting an aliquot of each PCR sample to electrophoresis at 100 V in 1.5% agarose gels, followed by ethidium bromide staining and visualization under UV illumination.

**RFLP and Southern blot analysis.** A 639-bp digoxigenin (DIG)-labeled cpe-specific DNA probe and a 318-bp DIG-labeled cpb2-specific DNA probe were prepared by a two-step PCR amplification method as previously described (9, 22). Isolated C. perfringens DNA samples, prepared as described previously (9, 22), were digested with NruI (New England Biolabs) (for cpe restriction fragment length polymorphism [RFLP] analysis), or with HpaI (New England Biolabs) (for cpb2 RFLP analysis), separated by electrophoresis on 1% agarose gels, and transferred by Southern blotting. The blots were hybridized with the DIG-labeled cpe or cpb2 probe, which was then detected using a DIG chemiluminescence detection system, utilizing CSPD [disodium 3-(4-methoxy-3-(4-methoxytricyclo[3,3.1.13.7]decane)-4-yl phenyl phosphate] "ready-to-use" substrate (Roche) as previously described (22, 23).

**PFGE Southern blot analysis.** Cells from overnight TGY cultures were collected by centrifugation and used to prepare agarose plugs containing genomic C. perfringens DNA as previously described (7, 8, 23, 25). The undigested total unsheared genomic DNA in 100 μl of an agarose plug was electrophoresed at 200 V with a Bio-Rad CHEF-DR II apparatus, with pulse times ramped from 5 to 120 s over 46 h at 150 V (6, 29). After PFGE, these gels were subjected to ethidium bromide staining and photographed under a UV transilluminator (Bio-Rad).

**PCR analyses for evaluating cpe-cpb2 linkage in cpe-cpb2 type A SD isolates.** Isolated C. perfringens DNA samples, prepared as described previously (9, 22), were subjected to PCR analyses using the battery of primers designed for amplification of 16 overlapping PCR fragments spanning the entire pF5603...
region between cpe and cbp2 as previously described (10). PCR products were sequenced on a 1% agarose gel and visualized using ethidium bromide staining.

**CPE Western blot analysis.** *C. perfringens* isolates were grown in TGG medium at 37°C overnight. A 0.2-ml aliquot of the TGG-grown culture was inoculated into 10 ml of Duncan-Strong (DS) sporulation medium (17) and allowed to grow at 37°C for 8 h. These DS cultures were sonicated until >95% of all cells were lysed.

From sonication, each culture lysate was analyzed for the presence of CPE by Western blot analysis using a CPE antibody as previously described (17, 22).

**Nucleotide sequencing of cbp2.** A 1,373-bp DNA fragment carrying the cbp2 open reading frame (ORF) and 262-bp upstream and 312-bp downstream sequences from 4178/94 or 5422/94 was PCR amplified using primers 5'GCTCTAAGGATATCFFAAATTTTAGACAAG-3' and 5'CCGAATTCATTCTTTTTAAGCTCATTTTTAATCTG-3' as previously described (29). The PCR products were then cloned into the pCR-XL-TOPO vector by using the TOPO XL cloning kit (Invitrogen). Both strands of the cbp2-containing DNA insert, from two clones for each isolate, were then sequenced using M13 forward and reverse primers.

**CPB2 Western blot analysis.** Culture supernatant fluid (CSF) was separated, by centrifugation at 500 × g from 20-ml TGY cultures of *C. perfringens* isolates grown for 18 h at 37°C. This CSF was used to isolate supernatant proteins by centrifugation at 500,000 × g in TGY broth at 37°C for 3 h, as previously described (9, 14), was separated by NaCl precipitation (29) or to concentrate to 20-fold at 4°C using Amicon Centriforc Ultra centrifugal filter devices (Millipore) (10). Prepared proteins were electrophoresed on a 0.1% sodium dodecyl sulfate (SDS)–12% polyacrylamide gel and transferred to a Sequi-Blot polyvinylidene difluoride membrane (Bio-Rad), and CPB2 Western blot analysis was performed as described previously (29).

**Northern blot analysis.** A 560-bp internal cbp2 DNA fragment was amplified from CWC245 by PCR using primer set 5'GGAGCAATAAGTCCAATGAA-3' and 5'TCCACATCCAATTTTTAATCTG-3' and labeled with alkaline phosphatase using the Gene Images AlkPhos direct labeling and detection system (Amersham Bioscience). Total RNA, isolated from *C. perfringens* strains grown in TGY broth at 37°C for 3 h as previously described (9, 14), was separated by electrophoresis on 1% agarose gels and Northern transferred. The blots were hybridized with the AlkPhos-labeled cbp2 probe, and hybridized probe was then detected by CDPstar chemiluminescence (Amersham Bioscience). The relative levels of cbp2 mRNA in surveyed SD isolates were determined from a calibration curve, which was made using various amounts (0.1 to 2.0 μg) of RNA prepared from a high-cpb2-producing pig strain, CWC245. The densitometric analysis curve, which was made using various amounts (0.1 to 2.0 μg) of RNA prepared from a high-cpb2-producing pig strain, CWC245. The densitometric analysis was performed on a Macintosh computer using the public domain NIH Image program (developed at the National Institutes of Health and available on the Internet at http://rsb.info.nih.gov/nih-image).

**RESULTS**

**Multiplex PCR toxin typing of *C. perfringens* fecal isolates from humans with SD.** A recent study (10) demonstrated that some *cpb* positive human isolates can carry *cbp2*. Therefore, we first subjected our collection of *cpb*-positive fecal isolates obtained from human cases of SD to a modified multiplex PCR analysis (11) to determine their toxin genotypes (A through E) and to demonstrate the presence of *cbp2* in these strains.

To ensure the reliability of multiplex PCR results, control PCRs were run using template DNA prepared from known *C. perfringens* type A, B, C, D, or E isolates. An ~324-bp PCR product matching the expected product size that should be amplified from the alpha-toxin gene (*cpa*) was observed using template DNAs prepared from all *C. perfringens* control strains, regardless of their toxin type (data not shown). These results are consistent with the known presence of *cpa* in *C. perfringens* isolates of all toxin types.

Additional PCR products of 196, 655, and 446 bp were amplified when known type C, D, or E isolates, respectively, were used as the source of template DNA for the multiplex PCR (data not shown). Those three PCR products match the expected sizes of products that the multiplex PCR assay should amplify from genes encoding beta toxin, epsilon toxin, or the iota-toxin A component, respectively.

When template DNA isolated from each of our 14 surveyed human SD isolates was subjected to multiplex PCR analysis, PCR products of 223 and 324 bp (but not the products of 196, 655, and 446 bp) were invariably obtained (see Fig. 1 for representative results). Therefore, these 14 SD isolates all represent isolates from *cpb*- and *cbp2*-negative isolates.

Two additional 223-bp and 567-bp products, which match the expected product sizes that should be amplified from *cpa* and *cbp2*, were also present when template DNA prepared from F5603, a known *cpa*-/*cbp2* type A isolate, was subjected to this same multiplex PCR analysis (Fig. 1). However, the *cpb2*-specific (567-bp) or *cpb2*-specific (223-bp) product was absent when multiplex PCR assay was performed on DNAs of F4969 (a *cpb2*+/*cbp2* type A human isolate) and JGS1807 (a *cpb2*/*cbp2* type A pig isolate), respectively. Finally, none of 223-bp and 567-bp products were obtained using template DNA prepared from ATCC 3624, a *cpb2*/*cbp2* type A isolate (Fig. 1). Collectively, these control results confirm the ability of the multiplex PCR assay to reliably distinguish *cpa*/*cbp2* isolates from *cpb*- and *cbp2*-negative isolates.

Additional PCR products of 196, 655, and 446 bp were amplified when known type C, D, or E isolates, respectively, were used as the source of template DNA for the multiplex PCR (data not shown). Those three PCR products match the expected sizes of products that the multiplex PCR assay should amplify from genes encoding beta toxin, epsilon toxin, or the iota-toxin A component, respectively.

When template DNA isolated from each of our 14 surveyed human SD isolates was subjected to multiplex PCR analysis, PCR products of 223 and 324 bp (but not the products of 196, 655, and 446 bp) were invariably obtained (see Fig. 1 for representative results). Therefore, these 14 SD isolates all clearly are classified as *cpa*−, type A isolates (Table 1). An additional PCR product of 567 bp was also obtained with DNAs of 12 out of 14 *cpa*+, SD isolates, classifying all 12 of these isolates as *cpa*−/*cbp2*+, type A isolates (Table 1).

**cpb2 RFLP genotyping of human SD isolates.** Our collection of SD isolates were then subjected to NruI RFLP Southern blot analysis for distinguishing between isolates carrying a chromosomal versus plasmid *cpa* gene (7). Previous results with this *cpb2* RFLP assay (7) have shown that the chromosomal
cpe is invariably present on an ~5-kb NruI-digested DNA fragment, while the plasmid cpe is always present on an NruI-digested DNA fragment of >20 kb. Southern blot analysis was first carried out with cpe-positive (NCTC8239 and F4969) and cpe-negative (ATCC 3624) C. perfringens isolates. As shown in Fig. 2, NruI-digested DNA from NCTC8239 produced a hybridizing band of ~5 kb, indicative of a chromosomal location of the cpe. However, NruI-digested DNA from F4969 produced a hybridizing band at >20 kb, indicative of a plasmid location of cpe. No hybridizing band was observed with DNA from cpe-negative human isolate ATCC 3624, indicating that our cpe RFLP Southern analysis was both reliable and specific.

When the same NruI RFLP Southern blot assay was applied to our collection of 14 cpe− SD isolates, the cpe probe produced a hybridizing band of >20 kb with DNAs of 12 isolates (see Table 1 and Fig. 2A for representative results), indicating that these 12 isolates all appeared to carry a plasmid cpe gene. For the remaining two SD isolates (6263/95 and 7458/94), the cpe probe hybridized to a 5-kb NruI DNA fragment, indicating that cpe is located on the chromosomes of these 2 isolates (see Table 1 and Fig. 2A for representative results).

cpb2 RFLP genotyping of SD isolates. Our multiplex PCR analyses demonstrated the presence of cpb2 in 12 of 14 surveyed cpe− SD isolates. To further ensure the presence of cpb2 in cpe− isolates, the SD isolates were subjected to cpb2 RFLP Southern blot analysis. Since previous studies (28, 29) successfully employed HpaI RFLP analyses to genotype cpb2+ animal GI disease isolates, SD isolates were genotyped using HpaI. Southern blot analysis was first carried out with cpb2-positive (CWC245) and cpb2-negative (106527) C. perfringens isolates. As expected from previous reports (28, 29), HpaI-digested DNA from CWC245 produced a hybridizing band of ~5 kb, and no hybridizing band was observed with HpaI-digested DNA from a cpb2-negative isolate, 106527. Interestingly, the cpb2 probe hybridized to a >20-kb HpaI DNA fragment of 12 SD isolates which were genotyped, using cpe RFLP analyses, as plasmid cpe isolates (see Table 1 and Fig. 2B for representative results). However, no cpb2-specific hybridizing band was obtained with HpaI-digested DNAs from two SD isolates (6263/95 and 7458/94) which were genotyped as chromosomal cpe isolates (Table 1). These results confirmed our multiplex PCR results that 12 out of 14 surveyed SD isolates carry both cpe and cpb2.

PFGE evidence supporting the chromosomal versus plasmid localization of cpe and cpb2 in C. perfringens type A isolates obtained from human SD. To further evaluate whether the cpe and cpb2 genes are located on the chromosome or the plasmid of the SD isolates, cpe+/cpb2+ C. perfringens SD isolates were subjected to PFGE Southern blot analysis. As shown in Fig. 3, no cpe-containing DNA from NCTC8239 entered the pulsed-field gel in the absence of restriction enzyme digestion, and a hybridizing band was produced only at the gel well (Fig. 3A). However, an ~360-kb cpe-containing DNA fragment did enter the gel if NCTC8239 was digested with I-CeuI prior to PFGE (note that the I-CeuI sites are located exclusively on the C. perfringens chromosome). These results are consistent with previous observations (7) that NCTC8239 carries a chromosomal cpe. In contrast, cpe-containing DNA from F4969 did migrate into the pulsed-field gel in the absence of restriction enzyme digestion (Fig. 3A), and the migration of this cpe-containing DNA was unaffected by I-CeuI treatment (data not shown), which is consistent with previous genotyping results (7) that cpe is located on a plasmid of F4969. When similar PFGE genotyping analyses were performed on undigested to-

**FIG. 2.** RFLP Southern blot analysis of SD isolates. (A) cpe RFLP Southern blot analysis of NruI-digested DNAs from SD isolates with a 639-bp DIG-labeled cpe-specific probe. Results are shown for control strains NCTC8239 (a chromosomal cpe, food poisoning isolate), F4969 (a plasmid cpe, non-food-borne GI disease isolate), and ATCC 3624 (a cpe-negative isolate) and for representative SD isolates 6253/94, 6257/94, 6263/95, and 7458/94. Molecular sizes of the hybridizing DNA fragments are given to the left. (B) cpb2 RFLP Southern blot analysis of HpaI-digested DNAs from SD isolates with a 318-bp DIG-labeled cpb2-specific probe. Results are shown for control isolates CWC245 (a cpb2-positive, pig GI disease isolate) and 106527 (a cpb2-negative isolate) and for representative SD isolates 6253/94, 6257/94, 3433/95, and 4178/94. Molecular sizes of the hybridizing DNA fragments are given to the left.

**FIG. 3.** PFGE evidence supporting the plasmid localization of cpe and cpb2 in SD isolates. (A) PFGE and Southern blot analysis of undigested (ud) and I-CeuI-digested (d) DNA from each of the C. perfringens isolates specified. Blots were probed with a 639-bp cpe-specific probe. Results are shown for control strains 8239 (NCTC8239; a chromosomal cpe, food poisoning isolate) and F4969 (a plasmid cpe, non-food-borne human GI disease isolate) and for representative SD isolates 7458/94, 6263/94, and 6257/94. The pulsed-field gel was calibrated with bacteriophage lambda DNA markers, whose migrations are shown at the right. (B) PFGE and Southern blot analysis of undigested DNA, prepared in agarose plugs, from each of the C. perfringens isolates specified. Blots were probed with a 318-bp cpb2-specific probe. Results are shown for control strains CWC245 (a cpb2-positive, type C isolate carrying cpb2 on a large plasmid) and for representative SD isolates 6253/94, 6257/94, and 3433/94. The pulsed-field gel was calibrated with bacteriophage lambda DNA markers, whose migrations are shown at the left.
the 16-overlapping-PCR strategy developed previously (10) on the same or different plasmids in our surveyed SD isolates, cpe carry a cpe plasmid-like sequences downstream of their isolates carrying the cpe plasmid with IS1470-like sequences downstream of cpe, where cpb2 is not present on the same cpe plasmid, produce PCR-amplified DNA products consistently only from reactions 9 to 11. When these 16 overlapping PCRs were performed on DNA of our control isolate F5603, where the cpe plasmid carries IS1151 sequences downstream of cpe and cpb2/cpb2 reside on the same plasmid (10), PCR products could be amplified from all 16 PCRs (data not shown), while DNA of control strain F4396, where the cpe plasmid carries IS1470-like sequences downstream of cpe and cpb2 reside on two different plasmids (10), produced PCR products only from reactions 9 to 11 (data not shown).

When these 16 PCRs were applied to DNAs of cpe+/cpb2+ C. perfringens SD isolates, 11 out of 12 surveyed isolates gave amplification patterns similar to those of F4396, suggesting that these isolates carry a cpe plasmid with IS1470-like sequences downstream of cpe and that cpe and cpb2 reside on two different plasmids. However, one strain (2728/94) gave amplification products similar to those of F5603, suggesting that this isolate’s cpe plasmid carries IS1151 sequences downstream of cpe and that both cpe and cpb2 are present on the same plasmid.

PFGE analysis for clonal relationship between cpe+/cpb2+ type A SD isolates. To examine the possibility that our surveyed C. perfringens type A SD isolates share a common clonal relationship with each other, total unsheared DNAs from representative isolates were digested with SmaI and subjected to PFGE. As shown in Fig. 4, PFGE analyses of SmaI-digested DNA identified no clonal relationship between the six SD isolates tested. In order to confirm that the results of the SmaI digestions were not due to uneven migration throughout the gel, further PFGE analyses were performed with MluI-digested DNA. The results of PFGE with MluI-digested DNA confirmed the lack of a clonal relationship between six surveyed SD isolates (data not shown).

CPE production by SD isolates. Next, we examined whether cpe+/cpb2+ C. perfringens type A SD isolates can produce CPE. When CPE Western blotting was performed with sporulating culture lysates from cpe-positive C. perfringens strains NCTC8239 and F4969 (Fig. 5A), an ~35-kDa immunoreactive band was obtained, which is consistent with previously reported size of the CPE protein (17). However, no cpe-specific immunoreactive band was detected in sporulating culture lysate of cpe-negative C. perfringens strain ATCC 3624 (Fig. 5A). When similar CPE Western blot analyses were performed on sporulating culture lysates prepared from SD isolates, all 14 surveyed isolates produced an ~35-kDa immunoreactive band that comigrated with the ~35-kDa band of strains NCTC8239 and F4969 (see Table 1 and Fig. 5 for representative results).

CPE production by cpe+/cpb2+ type A SD isolates. One of the key focuses of this study was to determine whether these newly identified cpe+/cpb2+ C. perfringens type A isolates can, in fact, produce CPEB. As expected, CPEB Western blotting analyses detected an ~28-kDa immunoreactive band in culture supernatant fluid prepared from vegetative and sporulating cultures of a known CPEB-producing C. perfringens strain,
CWC245 (Fig. 5B and data not shown). However, no CPB2 production was observed in CSF of either a vegetative or sporulating culture of a non-food-borne GI disease isolate and ATCC 28728/94, a cpe-negative isolate. The blot was developed by chemiluminescence detection to identify immunoreactive species. Results are shown for control strains F4969 (a plasmid cpe, non-food-borne GI disease isolate) and ATCC 3624 (a cpe-negative isolate) and for representative SD isolates 4178/94, 7458/94, and 3433/94. The arrow on the right indicates the migration of a CPE-specific immunoreactive band. (B) Culture supernatant of each sonicated culture lysate was then subjected to SDS-polyacrylamide gel electrophoresis followed by Western blotting with CPB2 antibodies. The blot was developed by chemiluminescence detection to identify immunoreactive species. Results are shown for control strains F4969 (a plasmid cpe, non-food-borne GI disease isolate) and ATCC 28728/94, a cpe-negative isolate, 106527 (a cpe-negative strain) and for representative SD isolates 4178/94, 7458/94, and 3433/94. The arrow at the left indicates the migration of a CPB2-specific immunoreactive band.

Comparative cpb2 Northern blot analysis of cpe+/cpb2+ SD isolates. A recent study (28) indicated that there is a positive correlation between cpb2 transcription levels and the amount of CPB2 produced by cpb2-positive C. perfringens isolates from horses with GI diseases. The differential production of CPB2 by human SD isolates in our current study (Fig. 5B) and by human AAD/SD isolates in a previous study (10) led us to test this hypothesis. We compared the levels of cpb2 transcription in low- to high-CPB2-producing SD isolates by Northern blot analysis. As expected, the cpb2-specific probe hybridized to an ~1.2-kb mRNA species present in total RNA prepared from a cpb2-positive strain, CWC245 (Fig. 6A). No hybridization signal was observed with total RNA extracted from a cpb2-negative isolate, 106527 (Fig. 6A), even though ethidium bromide staining (data not shown) confirmed that equivalent amounts of RNA samples from both CWC245 and 106527 had been electrophoresed. Using these Northern blot analyses, a single 1.2-kb band, which comigrated with the cpb2 probe-reactive band of CWC245, was detected in total RNAs of the SD isolates tested (Fig. 6). However, the expression of cpb2 was significantly lower in some SD isolates compared to that in SD isolate 4178/94 and in pig isolates JGS1807 and CWC245 (Fig. 6A and data not shown). Over three experimental repetitions, the 1.2-kb cpb2 probe-reactive species was reproducibly less abundant in RNA samples extracted from our three surveyed SD isolates, 2728/94, 4378/94, and 5422/94, than in RNA samples extracted from CWC245, 2728/94, 4378/94, and 5422/94. The cpb2 mRNA (1.2 kb) is indicated by an arrowhead. (B) Comparative expression of cpb2 mRNAs from representative SD isolates versus CWC245. Various amounts of RNAs extracted from CWC245, 2728/94, 4378/94, 3409/94, 4178/94, and 5282/94 cultures grown at 37°C for 3 h were subjected to Northern blot analysis using a 560-bp AlkPhos-labeled cpb2-specific probe. The relative levels of cpb2 mRNAs in SD isolates were determined as described in Materials and Methods. The cpb2 mRNA (1.2 kb) is indicated by an arrowhead. The various amounts of RNA loaded on the gel are indicated at the bottom.
and 5282/94 (Fig. 6). These results suggest that these five surveyed SD isolates produced very little 

cpb2 message, as would be consistent with Western blot results indicating low

(2728/94, 4828/94, and 3409/94) or nondetectable (5422/94 and 5282/94) CPB2 production by these SD isolates. When the relative 
cpb2 mRNA levels were determined by a semiquantitative Northern blot analysis, a 30-fold difference in cpb2 tran

scription was observed between an SD strain (4178/94) producing a high level of CPB2 and three SD strains (2728/94, 4828/94, and 3409/94) producing low levels of CPB2 (Table 2). These results suggest that the variation in the production of 

CPB2 by different SD isolates is mainly, if not completely, due to variation in the level of cpb2 transcripts.

**Nucleotide sequencing of cpb2 from SD isolates 5422/94 and 4178/94.** Our CPB2 Western blot and Northern blot results led us to hypothesize that the SD isolates 5422/94 and 5282/94 might carry a silent or atypical cpb2 (15). To evaluate this hypothesis, we determined the nucleotide sequence of cpb2 from 5422/94, which failed to produce CPB2, and from a high-

CPB2-producing strain, 4178/94. These analyses (data not shown) revealed that the cpb2 ORF sequences present in both 5422/94 and 4178/94 are intact; i.e., no mutations or termination codons were found in the ORFs. Strain 4178/94 had three nucleotide changes compared to CWC245 (12), i.e., A to G at position 100 (from ATG), A to G at position 206, and G to A at position 409. Strain 5422/94 had five nucleotide changes compared to CWC245, i.e., A to G at position 100, A to G at position 158, A to G at position 206, G to A at position 409, and A to G at position 677. These results suggest that our two surveyed SD isolates carry a “consensus,” but not “atypical,” cpb2 (15). Comparison of the cpb2 upstream and downstream sequences revealed that both up-

stream and downstream sequences are identical in 5422/94 and 4178/94 and match the previously determined cpb2 upstream and downstream sequences of CWC245 (12).

**Overexpression of cpb2 in F4969 by a recombinant plasmid carrying cpb2 from SD isolate 5422/94.** To confirm that the cpb2 ORF in 5422/94 is functional and that the lack of CPB2 production in 5422/94 was due to low expression of cpb2 mRNA, we performed complementation experiments with a recombinant plasmid pDR42, which carries the 5422/94 cpb2 ORF with its own promoter. Due to a low frequency of transformation (9), we failed

to introduce pDR42 into 5422/94 or 5282/94. However, we were able to introduce pDR42 into F4969, a highly transformable cpb2-negative AAD isolate. PCR analyses using cpb2-specific primers confirmed the presence of pDR42 in F4969 transformants, which were then named F4969(pDR42). When total RNA extracted from F4969(pDR42) was subjected to Northern blot analyses, a single 1.2-kb cpb2 probe-specific band was detected (Fig. 6A). No differences in the abundance of the 1.2-kb mRNA species were observed in RNA samples extracted from F4969(pDR42) and CWC245 (Fig. 6A), suggesting that the cpb2 present on the multicopy plasmid pDR42 could overexpress cpb2 mRNA in F4969. CPB2 Western blot analyses (Fig. 7) demonstrated that the plasmid pDR42 could overproduce CPB2 in F4969. pDR42 transformants were able to produce CPB2 in a secretion-dependent manner reminiscent of CPB2 production by CWC245; i.e., the pDR42 transformant produced no detectable CPB2-specific immunoreactivity in total cell proteins (data not shown) but did produce CPB2-specific immunoreactivity in culture supernatant proteins (Fig. 7). CPB2 Western blots also demonstrated, as expected, that CPB2 production (Fig. 7 and data not shown) was absent from both total cell and culture supernatant proteins when the shuttle vector pJIR751 alone was introduced into F4969, confirming that the observed overproduction of CPB2 in F4969(pDR42) is specifically caused by trans complementation of the wild-type cpb2 present in pDR42. Collectively, these results indicated that the cpb2 present in SD isolate 5422/94 is functional and that there is a positive correlation between cpb2 message levels and the amount of CPB2 produced by *C. perfringens* SD isolates.

**DISCUSSION**

The current study offers several significant contributions to our understanding of the pathogenesis of recently discovered cpe+/cpb2+ *C. perfringens* type A. First, most of our surveyed fecal isolates associated with cases of human SD were identi

fied as cpe+/cpb2+ *C. perfringens* type A. Among 14 of these surveyed SD isolates, 12 carry their cpe gene on the plasmid
and 2 carry it on the chromosome. The presence of cphb2 only in C. perfringens plasmid cpe isolates and not in chromosomal cpe isolates significantly strengthens the hypothesis (10) that CPB2 can be an accessory toxin in human GI diseases caused by C. perfringens isolates carrying plasmid cpe.

Second, our cphb2 PFGE/Southern blot results for 12 of the cpe−/cphb2− type A British human SD isolates indicate that all of these isolates carry their cphb2 gene on a large plasmid. When these results are coupled with previous results (10, 12, 28, 29), it is now clear that the cphb2 gene has a plasmid location in C. perfringens isolates, regardless of their toxinothypes or origins of isolation. The battery of 16 PCR results demonstrated that cpe and cphb2 reside on different plasmids in 11 of 12 surveyed cpe−/cphb2− SD isolates. Furthermore, PFGE/RFLP analyses performed in this study demonstrated no clonal relationship between the surveyed SD isolates. Collectively, these results suggest that the acquisition of the cphb2 and/or cpe plasmid may be a critical step for C. perfringens type A isolates to become enteropathogenic for humans. Further studies are needed to determine whether the cphb2 plasmid, like the cpe plasmid (4), can be mobilized between C. perfringens isolates.

Third, our present study provides an array of evidence supporting that all of our surveyed SD isolates carry functional cpe and cphb2 and that the lack of CPB2 production in two SD isolates was not due to the presence of silent or atypical cphb2, as follows. (i) Western blot analyses demonstrated the production of CPE by all 12, and of CPB2 by 10 of the 12, cpe−/cphb2− SD isolates. However, a significant variation in the level of CPB2 production was observed between SD isolates. A similar variability in the level of CPB2 production by human AAD/SD isolates was also previously observed (10). (ii) Nucleotide sequencing identified an intact cphb2 ORF in our surveyed CPB2 Western blot-negative SD isolate 5422/94. (iii) Complementation studies demonstrated that a recombinant plasmid carrying 5422/94 cphb2 induced CPB2 production in F4969 (a naturally cphb2-negative strain) (Fig. 7), indicating that the 5422/94 cphb2 is fully functional. Collectively, these results suggested that the different levels of CPB2 production observed in our surveyed human SD isolates may be due to differences in cphb2 transcription levels.

Finally, the most significant finding of this study is the presentation of evidence for a transcriptional regulation of cphb2 in human SD isolates, as was observed previously in cphb2+ C. perfringens type A isolates from horses with GI diseases (28). A 30-fold-lower cphb2 mRNA level was observed in our surveyed low-CPB2-producing human SD isolates compared to that in the high-CPB2-producing isolate 4178/94 (Table 2). This differential level of mRNA synthesis might be a major cause of differential CPB2 production by human SD isolates in our current study and by human AAD/SD isolates in a previous study (10). This received further support from our observation that the complementing strain F4969(pDR42), which produced significantly higher levels of mRNA (Fig. 6A), produced CPB2 at a level similar to that of CWC245 or 4178/94 (Fig. 7 and data not shown). These findings provide a genetic basis, at least in part, of the lack of a detectable level of CPB2 production in vitro in 5422/94, which may also be applicable to SD isolate 5282/94 and two previously identified cpe−/cphb2− human GI disease isolates that failed to produce CPB2 (10). Our complementation studies also suggested that there is a positive correlation between cphb2 transcription levels and the amount of CPB2 produced by C. perfringens and that decreased transcription and/or message instability may be involved, at least in part, in the lower CPB2 production noted for some human SD isolates compared to the pig isolate CWC245. However, no degraded cphb2 transcript was detected on the Northern blots of our surveyed SD isolates (Fig. 6), suggesting that the difference in CPB2 production in vitro between human SD isolates may have arisen due to different rates of cphb2 transcription. The differential level of cphb2 transcription in C. perfringens was previously reported; the level of cphb2 transcripts differed by 35-fold between horse and pig GI disease isolates, and the greater production of CPB2 by the pig GI disease isolates is mainly due to an increased level of cphb2 transcripts (28). However, it is not yet clear what could be responsible for the lower rate of cphb2 transcription in horse GI disease isolates (28) and in human SD isolates (this study) compared to that in pig GI disease isolates. The 1,373-bp sequenced fragment from human SD isolates does not contain any features which would account for different cphb2 expression between the pig GI disease isolate CWC245 and our surveyed human SD isolates. The cphb2 upstream and downstream regions in human SD isolates are identical to those in the pig isolate CWC245. Therefore, further research is necessary to identify the relevant difference(s) that might be present outside of this 1,373-bp sequenced fragment and be involved in regulating cphb2 expression.

The association of cpe−/cphb2− C. perfringens type A isolates with human SD in England raises an important but still unanswered question. Is production of CPE enough to cause GI symptoms of CPE-associated human SD, or do these symptoms also result from the production of CPB2? Our present study raises the possibility that the high-level expression of cphb2 in vivo by cpe−/cphb2− C. perfringens type A might contribute to the GI symptoms. Since the current study confirms that cpe−/cphb2− SD isolates can produce both CPE and CPB2, it is possible that CPB2 is an accessory toxin contributing to the pathogenesis of diseases caused by these isolates. Further cpe/cphb2 knockout studies should fully address the relative contributions of CPE versus CPB2 in C. perfringens type A-associated GI diseases in humans.

ACKNOWLEDGMENTS

This research was supported by a grant from the N. L. Tartar Foundation of Oregon State University, by a grant from the Medical Research Foundation of Oregon Health Science University, by a grant from the Agricultural Research Foundation of Oregon State University, and by USDA grant 2002-02281 from the Ensuring Food Safety Research Program (all to M.R.S.).

We are grateful to B. A. McClane, University of Pittsburgh School of Medicine, for providing us with CPE antibody. We thank Nahid Mahfuz for technical assistance and I-Hsiu Huang for editorial comments.

REFERENCES


