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Evidence That the Enterotoxin Gene Can Be Episomal in *Clostridium perfringens* Isolates Associated with Non-Food-Borne Human Gastrointestinal Diseases

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***Clostridium perfringens* enterotoxin (CPE) is responsible for the diarrheal and cramping symptoms of human *C. perfringens* type A food poisoning. CPE-producing *C. perfringens* isolates have also recently been associated with several non-food-borne human gastrointestinal (GI) illnesses, including antibiotic-associated diarrhea and sporadic diarrhea. The current study has used restriction fragment length polymorphism (RFLP) and pulsed-field gel electrophoresis (PFGE) analyses to compare the genotypes of 43 *cpe*-positive *C. perfringens* isolates obtained from diverse sources. All North American and European food-poisoning isolates examined in this study were found to carry a chromosomal *cpe*, while all non-food-borne human GI disease isolates characterized in this study were determined to carry their *cpe* on an episome. Collectively, these results provide the first evidence that distinct subpopulations of *cpe*-positive *C. perfringens* isolates may be responsible for *C. perfringens* type A food poisoning versus CPE-associated non-food-borne human GI diseases. If these putative associations are confirmed in additional surveys, *cpe* RFLP and PFGE genotyping assays may facilitate the differential diagnosis of food-borne versus non-food-borne CPE-associated human GI illnesses and may also be useful epidemiologic tools for identifying reservoirs or transmission mechanisms for the subpopulations of *cpe*-positive isolates specifically responsible for CPE-associated food-borne versus non-food-borne human GI diseases.**

Clostridium perfringens type A food poisoning currently ranks as the second most common foodborne disease in the United States (2). The diarrhea and cramps that comprise the typical clinical symptoms of this human illness are induced by a single 35-kDa polypeptide named *C. perfringens* enterotoxin (CPE) (20, 23, 24). Although *cpe*-positive isolates represent only a very small fraction of the global *C. perfringens* population (16, 19, 31, 32), recent epidemiologic studies (5–8, 10, 17, 22, 26) indicate that these bacteria can also cause non-food-borne gastrointestinal (GI) illnesses in humans. In particular, some surveys (5, 10, 26) have suggested that CPE-producing *C. perfringens* may be responsible for ~10% of all cases of antibiotic-associated diarrhea (AAD) and 5 to 20% of all cases of sporadic non-food-borne diarrhea (SPOR). Some evidence implicating CPE-producing *C. perfringens* as a cause of AAD and SPOR includes the following. (i) Feces from many humans suffering from AAD (5, 7, 22) or SPOR (10, 17, 26) contain CPE at levels comparable to those found in feces from *C. perfringens* type A food-poisoning victims, while CPE is rarely, if ever, detectable in feces from either healthy individuals or individuals suffering from gastroenteritis caused by other enteropathogens (1, 3, 27); (ii) in the absence of other known enteropathogens, unusually high levels of *C. perfringens* cells or spores are present in feces from many individuals suffering from AAD or SPOR (7, 10); and (iii) many *C. perfringens* strains isolated from the feces of individuals suffering from AAD or SPOR are able to express CPE (7, 9), in contrast to the lack of expression of CPE by nearly all *C. perfringens* isolates found in feces from either healthy individuals or individuals suffering from gastroenteritis caused by other pathogens (27, 28).

Genotypic differences have recently been identified among *cpe*-positive *C. perfringens* isolates. Results from two studies (13, 18) indicate that at least some *C. perfringens* type A food-poisoning isolates carry *cpe* on their chromosomes and that at least some *cpe*-positive *C. perfringens* veterinary isolates carry *cpe* on an episome (note that *cpe*-positive *C. perfringens* isolates are also considered important causes of enteric disease in domestic animals [30]). However, these putative source-related genotypic associations remain tentative due to the relatively small number and limited (European) geographic origin of enterotoxigenic *C. perfringens* isolates genotyped to date.

To better appreciate the role that genotypically distinct subpopulations of enterotoxigenic *C. perfringens* may play in CPE-associated human (and veterinary) diseases, the present study has genotypically characterized >40 *cpe*-positive *C. perfringens* isolates. Notably, the isolates used in our study originated from considerably more diverse host, geographic, and disease sources than the *cpe*-positive isolates genotyped to date and include a sizeable number of *cpe*-positive non-food-borne human GI disease isolates which had not yet been genotypically characterized. Results from the current study indicate that most (or all) *cpe*-positive food-poisoning isolates, regardless of their isolation date or geographic origin, carry a chromosomal *cpe*. Furthermore, our study also presents the first evidence that *cpe* is episomal in many (or all) CPE-associated non-food-borne human GI disease isolates. These new findings hold potential epidemiologic significance for suggesting that genotypically distinct subpopulations of *cpe*-positive *C. perfringens* isolates may be responsible for human *C. perfringens* type A food poisoning and CPE-associated non-food-borne human GI diseases.

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MATERIALS AND METHODS

Bacterial strains. The *C. perfringens* isolates used in this study are listed and described in Table 1.

Preparation of a DIG-labeled *cpe*-specific probe. A 639-bp digoxigenin (DIG)-labeled, double-stranded DNA probe corresponding to internal *cpe* sequences was prepared by a two-step PCR amplification method, as described previously (14).

Restriction fragment length polymorphism (RFLP) analysis. Starter vegetative *C. perfringens* cultures were grown overnight at 37°C in fluid thioglycolate broth (FTG) (Difco), as described previously (15). A 500-μl aliquot of each FTG starter culture was then used to inoculate 10 ml of TGY broth (3% Trypticase, 2% glucose, 1% yeast extract, 0.1% cysteine [14]), and these TGY cultures were incubated for 8 h at 37°C.

A previously described protocol (14) was used to isolate total *C. perfringens* DNA from each TGY culture. Samples of each isolated DNA were then digested to completion with either *Nru*I (New England BioLabs) or *Eco*RI (Boehringer Mannheim), according to the manufacturer's specifications. Eight micrograms of the digested DNA was then subjected to conventional electrophoresis on 0.8% agarose gels, transferred to positively charged nylon membranes (Nytran Maximum Strength; Schleicher & Schuell), and fixed with UV light (29). The 639-bp DIG-labeled *cpe* gene probe, prepared as indicated above, was hybridized to these blots, as described previously (14), and the blots were washed at high stringency (29). Hybridized probe was detected with a DIG chemiluminescence detection system with Lumi-Phos 530 substrate (Boehringer Mannheim).

PFGE studies. *C. perfringens* cultures were grown to the mid-exponential phase (i.e., for approximately 5 h) at 37°C in TGY broth. Cells from these TGY broth cultures were used to prepare genomic *C. perfringens* DNA in agarose plugs, as described previously (12). DNA in 40 μl of an agarose plug was then incubated overnight at 37°C with or without 1.6 U of I-*Ceu*I (New England Biolabs) in 200 μl of the buffer solution recommended by the enzyme manufacturer. Following these incubations, the DNA samples were analyzed by pulsed-field gel electrophoresis (PFGE) with 0.7% agarose gels prepared with PFGE-grade agarose (Bio-Rad). PFGE was performed with a Bio-Rad CHEF-DR II apparatus, with pulse times ramped from 20 to 120 s over 12 h, followed by ramping from 60 to 100 s over 12 h (21). After PFGE, the gels were subjected to Southern analysis by using the same procedure described above for DNA samples subjected to conventional electrophoresis, except that the PFGE blots were washed under standard stringency conditions (4).

The rationale for these PFGE analyses, which have been used previously to genotype *cpe*-positive *C. perfringens* isolates (13, 18), is that, without any restriction enzyme digestion, unsheared *C. perfringens* chromosomal DNA is too large to enter a pulsed-field gel. However, because of its smaller size, at least some episomal DNA should enter a pulsed-field gel, even without any restriction enzyme treatment. Furthermore, since all I-*Ceu*I sites are chromosomal in *C. perfringens* DNA (13, 18), I-*Ceu*I digestion of samples should produce chromosomal DNA fragments that can enter pulsed-field gels, but treatment with this restriction enzyme should not effect the migration of episomal DNA. Consequently, if isolates with a chromosomal *cpe* are analyzed by this PFGE protocol, their *cpe*-containing DNA should remain in the gel wells when samples are not treated with I-*Ceu*I, but some *cpe*-containing DNA from these isolates should enter the gel when samples are treated with I-*Ceu*I. In contrast, PFGE analysis of isolates carrying an episomal *cpe* should show similar migration of *cpe*-containing DNA into gels, whether or not a sample is treated with I-*Ceu*I.

RESULTS

Comparative RFLP analysis of *cpe*-positive isolates. A study (13) recently reported that *cpe* is localized to 5-kb *Nru*I and 10-kb *Eco*RI DNA fragments in several European enterotoxigenic food-poisoning isolates but is present in several European veterinary isolates on DNA of >20 kb following digestion with either *Nru*I or *Eco*RI. Considering that only a limited number of isolates, all with similar origins, were examined in that previous study (13), in our current study we initially tested these putative source-related *cpe* RFLP patterns using our laboratory's collection of *cpe*-positive *C. perfringens* isolates.

Our initial *cpe* RFLP experiments confirmed a previous report (13) that European food-poisoning isolates NCTC 8239, NCTC 8798, and NCTC 10239 all carry *cpe* on 5-kb *Nru*I and 10-kb *Eco*RI fragments (see Fig. 1 for representative RFLP blot results for NCTC 8239 and NCTC 10239 and Table 1 for a summary of our results for all three of these strains). When similar RFLP analyses were extended to DNA from four European food-poisoning isolates and eight North American food-poisoning isolates that had not been previously genotyped, *cpe* was found to be similarly localized (see Fig. 1 for representative RFLP blot results for previously unexamined strains NCTC 8238, NCTC 8359, NCTC 8799, FD 1041, and C-1841 and Table 1 for a summary of our RFLP results for all 12 previously unexamined food-poisoning isolates) to 5-kb *Nru*I and 10-kb *Eco*RI DNA fragments.

The *cpe* RFLP profiles reported for food-poisoning isolates in Table 1 (and elsewhere [13]) differ from those reported previously (13) for several veterinary isolates, all of which were from Europe. To confirm that the *cpe* RFLP profiles of *cpe*-positive veterinary isolates consistently differ from those of food-poisoning isolates, we next performed *cpe* RFLP analyses with 10 *cpe*-positive veterinary isolates that had not been previously genotypically characterized (Table 1). These RFLP analyses revealed that all nine of the North American veterinary isolates in our collection share similar *Nru*I or *Eco*RI *cpe* RFLP patterns (see Fig. 1 for representative RFLP blot results for veterinary isolate 452 and Table 1 for a summary of our RFLP results for all nine North American veterinary isolates examined), with *cpe* always localizing to DNA of >20 kb following either *Nru*I or *Eco*RI digestion. While the RFLP patterns of these North American veterinary isolates match those reported previously (13) for European veterinary isolates, our study has also identified the first veterinary isolate with an atypical *cpe* RFLP pattern. European veterinary isolate 29, which was reputedly obtained from a diarrheic pig, was observed to carry its *cpe* on a 5-kb *Nru*I and a 10-kb *Eco*RI fragment (Table 1).

Having confirmed, with the single exception of isolate 29, that food-poisoning and veterinary enterotoxigenic *C. perfringens* isolates exhibit consistent differences in their *cpe* RFLP patterns, we next performed the first *cpe* RFLP analyses of enterotoxigenic isolates originating from patients with CPE-associated non-food-borne human GI diseases. Interestingly, these RFLP analyses (see Fig. 1 for representative RFLP blot results for isolates B11, B45, F4591, and F4969 and Table 1 for a summary of our RFLP results for all non-food-borne human GI disease isolates examined) demonstrated that, following either *Nru*I or *Eco*RI digestion, *cpe* is present on DNA fragments of >20 kb for all 7 AAD isolates and all 11 SPOR isolates in our collection. Therefore, these results demonstrate that the non-food-borne human GI isolates in our collection consistently exhibit *cpe* RFLP patterns different from those of human food-poisoning isolates.

Finally, the specificity of the *cpe* probe used in the current RFLP studies has been confirmed by control studies demonstrating (Table 1) that this probe does not hybridize to either *Nru*I- or *Eco*RI-digested DNA from *C. perfringens* ATCC 3624, a strain previously shown to be *cpe* negative (14, 25).

PFGE confirmation of episomal versus chromosomal locations for *cpe* in selected isolates. The RFLP results presented above supporting source-related genotypic differences between *cpe*-positive *C. perfringens* isolates are particularly interesting in light of recent studies (13, 18) showing that *cpe* is chromosomal in several European food-poisoning isolates carrying *cpe* on 5-kb *Nru*I and 10-kb *Eco*RI fragments but is present on an episome in several European veterinary isolates carrying *cpe* on DNA fragments of >20 kb after *Nru*I or *Eco*RI digestion. Therefore, the RFLP results shown in Fig. 1 and Table 1 apparently suggest that all food-poisoning isolates in our collection carry a chromosomal *cpe* and that all non-food-borne human GI disease and veterinary (except isolate 29) isolates in our collection carry *cpe* on an episome.

To test this hypothesis, selected isolates in our collection were analyzed by the same PFGE approach (see Materials and Methods) used in previous studies (13, 18) to establish chromosomal or episomal locations for *cpe* in selected European

TABLE 1. Summary of genotypic characterization results obtained in this study for *cpe*-positive *C. perfringens* isolates

Isolate group and source	Strain (reference)	*cpe* RFLP pattern (kb) obtained with the following endonuclease:		PFGE-determined *cpe* location[a]
		*Eco*RI	*Nru*I	
cpe-negative strain	ATCC 3624 (19)	Negative	Negative	Negative
Food-borne disease isolates[b]				
1950s European food-poisoning isolates	NCTC 8235 (19)	10	5	ND
	NCTC 8238 (19)	10	5	ND
	NCTC 8239 (19)	10	5	S
	NCTC 8359 (19)	10	5	ND
	NCTC 8798 (19)	10	5	ND
	NCTC 8799 (19)	10	5	ND
	NCTC 10239 (19)	10	5	Chromosomal
1980s North American food-poisoning isolates	C-1841 (3)	10	5	Chromosomal
	C-1849 (3)	10	5	ND
	C-1851 (3)	10	5	ND
	C-1869 (3)	10	5	ND
	C-1881 (3)	10	5	ND
	C-1887 (3)	10	5	ND
	FD 1041 (R. Labbe)	10	5	Chromosomal
1990s North American food-poisoning isolate	5 (19)	10	5	ND
Non-food-borne disease isolates				
Stool isolates from 1980s European antibiotic-associated diarrhea study[c]	B2 (22)	>20	>20	Episomal
	B11 (22)	>20	>20	Episomal
	B38 (22)	>20	>20	Episomal
	B41 (22)	>20	>20	ND
	B45 (22)	>20	>20	ND
	Newbury 16 (P. Borriello)	>20	>20	Episomal
Stool isolates from 1990s European SPOR study[d]	F4013 (M. Brett)	>20	>20	ND
	F4129 (M. Brett)	>20	>20	Episomal
	F4393 (M. Brett)	>20	>20	ND
	F4396 (M. Brett)	>20	>20	ND
	F4406 (M. Brett)	>20	>20	ND
	F4591 (M. Brett)	>20	>20	Episomal
	F4859 (M. Brett)	>20	>20	ND
	F4969 (M. Brett)	>20	>20	Episomal
	F5537 (M. Brett)	>20	>20	ND
	F5599 (M. Brett)	>20	>20	ND
	F5603 (M. Brett)	>20	>20	Episomal
Veterinary isolates				
1990s European veterinary isolate from pig with enteritis	29[e] (D. J. Taylor)	10	5	S
1990s North American veterinary isolates	153[e] (19)	>20	>20	ND
	157 (19)	>20	>20	ND
	222[e] (19)	>20	>20	Episomal
	382 (19)	>20	>20	ND
	452[e] (19)	>20	>20	Episomal
	455[e] (19)	>20	>20	ND
	456[e] (19)	>20	>20	ND
	457[e] (19)	>20	>20	ND
	458[e] (19)	>20	>20	Episomal

[a] ND, not determined; S, smearing of DNA bands observed (see Results).
[b] Note that in reference 19, NCTC 8235 is called 182, NCTC 8238 is called 176, NCTC 8359 is called 175, and NCTC 8799 is called 183.
[c] Note that these isolates, belonging to several different capsular serotypes, were all obtained from CPE-positive feces of diarrheic humans.
[d] Note that these isolates were all obtained form CPE-positive feces of humans suffering from isolated cases of sporadic diarrhea.
[e] Note that these isolates were obtained form diarrheic domestic animals.

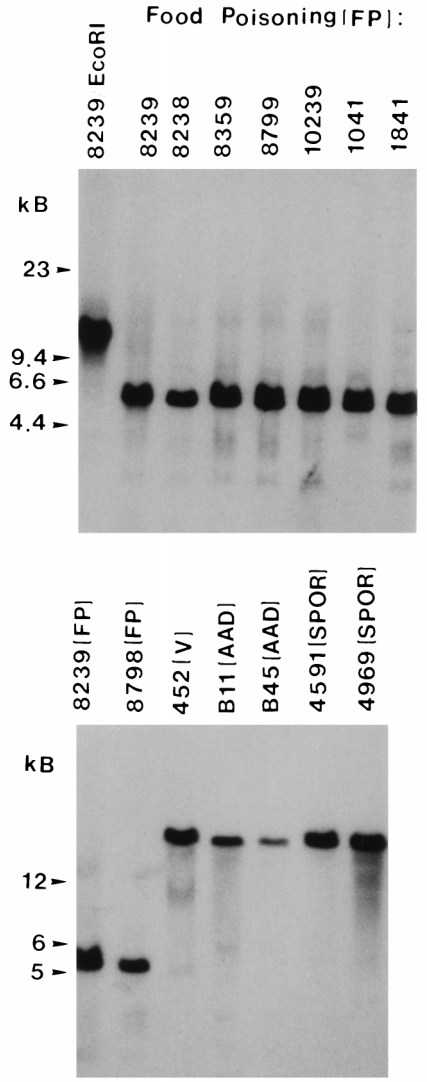

FIG. 1. RFLP analysis of *Nru*I-digested total DNA from selected *C. perfringens* disease isolates demonstrating presumptive evidence for episomal versus chromosomal distribution of *cpe* in isolates from different disease sources. Southern blots were probed with a 639-bp DIG-labeled *cpe*-specific fragment. VET, veterinary. All samples except 8239/*Eco*RI were cut with *Nru*I; 8239/*Eco*RI was cut with *Eco*RI and the results are shown for comparison. Molecular sizes (in kilobase pairs) of the DNA markers are given to the left of each blot. See Table 1 for full strain designations.

veterinary and food-poisoning isolates. When we performed these PFGE analyses with food-poisoning isolate NCTC 10239, no migration of *cpe*-containing DNA into pulsed-field gels was detected in the absence of I-*Ceu*I treatment (data not shown). However, an ~360-kb *cpe*-containing restriction fragment was observed when DNA from this food-poisoning strain was treated with I-*Ceu*I prior to PFGE (data not shown). These results are in complete agreement with previous PFGE characterization of this strain (13, 18). When the same PFGE analyses were extended to North American food-poisoning isolates FD 1041 (see Fig. 2 and Table 1) and C-1841 (Table 1), which had not previously been examined by PFGE, the same migration patterns were observed for the *cpe*-containing DNAs of these two strains, as reported above for *cpe*-containing DNA from NCTC 10239. Therefore, our PFGE results confirm that all three of these food-poisoning isolates carry a chromosomal *cpe*.

The same PFGE analyses described above were also performed with DNAs from North American veterinary isolates 222, 452, and 458, which also have not been previously analyzed by PFGE, in order to confirm our RFLP results suggesting that these isolate carry an episomal *cpe*. PFGE analyses of DNAs from these three North American veterinary isolates showed that their *cpe*-containing DNA migrates into pulsed-field gels in the absence of I-*Ceu*I treatment. Further supporting the fact that *cpe* is episomally localized (see Materials and Methods) in these three isolates, we observed no change in the migration of *cpe*-containing DNA from isolates 222, 452, and 458 with I-*Ceu*I treatment (see Fig. 2 for representative PFGE blot results for isolate 452 and Table 2 for a summary of PFGE conclusions for all three veterinary isolates examined). During these studies we also attempted to perform similar PFGE analyses with DNA from isolate 29, the unusual veterinary isolate that exhibits an RFLP profile suggesting a chromosomal *cpe* gene. Unfortunately, this strain was not amenable to PFGE analyses, yielding DNA smears on PFGE gels even in the absence of treatment with I-*Ceu*I (data not shown). Similar PFGE smearing has been observed (Table 1) (13) for food-poisoning strain *C. perfringens* NCTC 8239 and is believed to result from the fact that some *C. perfringens* strains produce unusually large amounts of a very stable endogenous nuclease(s) (12).

The final series of experiments conducted in the current study involved extending PFGE analyses to eight representative non-food-borne human GI disease isolates from our collection (including four AAD and four SPOR isolates, none of which have previously been examined by PFGE). Interestingly, DNAs from these eight non-food-borne human GI disease isolates were found to exhibit the same PFGE behavior described above for DNAs from veterinary isolates 222, 452, and 458, which carry an episomal *cpe*. That is, we observed the entrance of *cpe*-containing DNA species from all eight of these AAD or SPOR isolates into pulsed-field gels in the absence of I-*Ceu*I treatment, and the migration of these *cpe*-containing species was unaffected by I-*Ceu*I treatment (see Fig. 2 for representative PFGE blot results for isolates B11 and F4969 and Table 2 for a summary of PFGE results for all 8 non-food-borne disease isolates examined in this study).

Finally, the specificity of the *cpe* probe used in our PFGE studies was confirmed by demonstrating that this probe did not hybridize to either nondigested (Fig. 2) or I-*Ceu*I-digested (data not shown) samples of DNA from *cpe*-negative strain ATCC 3624 that had been run on pulsed-field gels.

DISCUSSION

The current study offers several significant contributions to our understanding of the recently discovered (13, 18) subpopulations of *cpe*-positive *C. perfringens*. First, the RFLP results presented in this study for three previously examined European food-poisoning isolates (NCTC 8239, NCTC 8798, and NCTC 10239) and four previously unexamined European food-poisoning isolates (NCTC 8235, NCTC 8238, NCTC 8359, and NCTC 8799), which are supported by PFGE results for NCTC 10239, significantly strengthen the hypothesis (13, 18) that most, if not all, European food-poisoning isolates carry a chromosomal *cpe*. Our RFLP results confirming that NCTC 8235 is *cpe* positive are particularly interesting. Even though NCTC 8235 was originally isolated as an enterotoxigenic strain, a recent study (13) could not detect *cpe* in ATCC 12922, which is reputedly the same strain as NCTC 8235. Col-

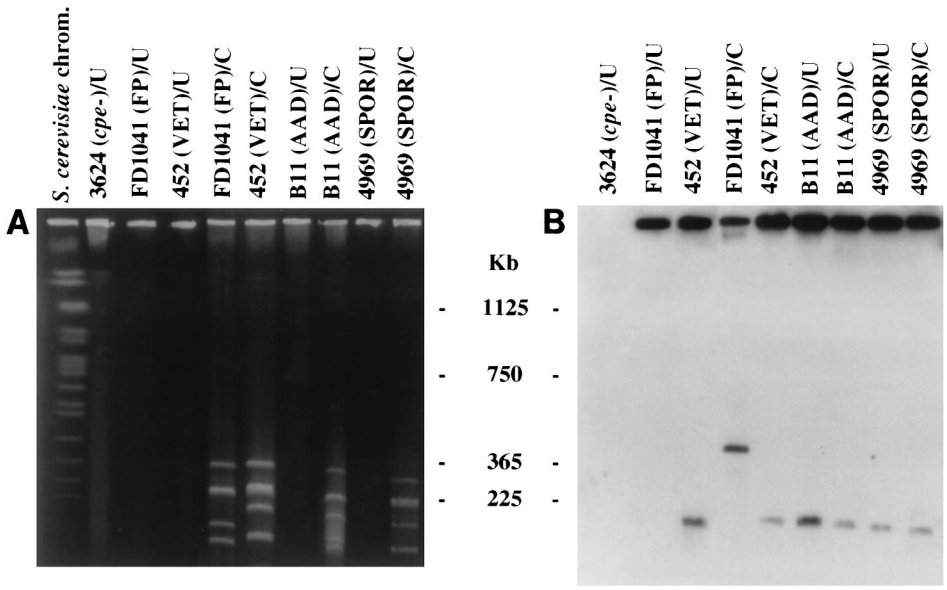

FIG. 2. PFGE evidence supporting the localization of *cpe* to episomes in isolates from some disease sources. PFGE and Southern hybridization analysis of undigested and I-*Ceu*I-digested genomic DNA from select *C. perfringens* disease isolates. (A) pulsed-field gel stained with ethidium bromide. (B) Southern blot of the gel shown in panel A. The blot was probed with a 639-bp DIG-labeled *cpe*-specific fragment. FP, food poisoning; VET, veterinary; U, undigested, intact genomic DNA; C, I-*Ceu*I-digested DNA. The gel was calibrated with *Saccharomyces cerevisiae* chromosomal DNA. Molecular sizes of the DNA markers are given in the center. See Table 1 for full strain designations.

lectively, these results for NCTC 8235/ATCC 12922 could be indicating that the *cpe* gene disappeared from some subcultures of NCTC 8235. If so, these subcultures may have lost a mobile *cpe*-containing genetic element that had been integrated into the NCTC 8235 chromosome, since a putative *cpe*-carrying transposon has recently been identified in the chromosome of NCTC 8239, another European food-poisoning strain (11).

The current study also reports the first genotypic characterization of *cpe*-positive North American food-poisoning isolates. Our RFLP results for eight North American food-poisoning isolates, supported by PFGE results for North American food-poisoning isolates FD1041 and C-1841, indicate that all these isolates (which originated from three separate food-poisoning outbreaks) also carry a chromosomal *cpe*. When these results are coupled with similar results for European food-poisoning isolates ((13, 18); this study), it is now clear that *cpe* has a chromosomal location in most (or all) *C. perfringens* food-poisoning isolates, regardless of the isolate's geographic origin or isolation date (note that the food-poisoning isolates examined in our current study and previous studies [13, 18] were isolated over a period extending from the 1950s to the 1990s).

Additionally, the PFGE and RFLP results presented in the current study provide the first evidence that most (or all) *cpe*-positive North American veterinary isolates carry an episomal *cpe*. When combined with previous genotyping results (13, 18) for European *cpe*-positive veterinary isolates, it now appears that most *cpe*-positive veterinary isolates, regardless of geographic origin or date of isolation, carry an episomal *cpe*. However, the current study has also identified the first anomalous *cpe*-positive veterinary isolate (isolate 29) whose DNA does not fit the standard genotypic pattern for these veterinary isolates, i.e., RFLP analyses suggest that isolate 29 is an unusual *cpe*-positive veterinary isolate carrying a chromosomal *cpe*. Since isolate 29 is a porcine isolate, this observation could provide an insight into the epidemiology of *C. perfringens* type A food poisoning by suggesting that some cases of *C. perfringens* type A food poisoning, which almost always results from consumption of a contaminated meat product (22), may be caused by ingesting meats originating from animals that had been contaminated with atypical *C. perfringens* veterinary isolates (like isolate 29) carrying the chromosomal *cpe* linked to food poisoning.

The current study's most significant finding is presentation of the first evidence that *cpe* is episomal in many or all *cpe*-positive isolates originating from CPE-associated non-food-borne human GI diseases. While this association is based upon PFGE analysis of eight different non-food-borne human GI disease isolates (and is further implied by the similar RFLP patterns shared by these 8 isolates analyzed by PFGE and the 10 other non-food-borne human GI disease isolates examined in the current study), caution should still be exercised before extrapolating the overall involvement of isolates with episomal *cpe* in CPE-associated non-food-borne human GI diseases from the current data. Since the AAD and SPOR isolates used in our current study all share a similar (European) geographic origin, it will be important to conduct additional surveys with isolates from other geographic locations to confirm the putative association between episomal *cpe* isolates and CPE-associated non-food-borne human GI diseases suggested by our study. In this respect, it should be noted that reliably characterized North American enterotoxigenic AAD and SPOR *C. perfringens* isolates were unavailable for our current study, reflecting the limited research attention CPE-associated non-food-borne human GI illnesses have received to date in the United States.

Pending results from additional surveys, the fact that all 18 non-food-borne human GI disease isolates surveyed in the current study apparently carry an episomal *cpe* still holds considerable significance. First, this result provides important new evidence that enterotoxigenic *C. perfringens* isolates are, in fact, responsible for non-food-borne human GI diseases. Although this association has been strongly suggested by classical epide-

miologic studies (see Introduction), these previous studies have not totally eliminated the possibility that individuals putatively identified as suffering from CPE-associated AAD or SPOR were, instead, victims of isolated, unrecognized cases of *C. perfringens* type A food poisoning. However, if these individuals had been sickened by *C. perfringens* type A food poisoning, then it would be expected that the "non-food-borne human GI disease" isolates in our collection should have exhibited the same genotypic patterns as isolates causing recognized cases of *C. perfringens* type A food poisoning.

Second, genotyping results for our non-food-borne disease isolates now strongly suggest that both episomal and chromosomal *cpe*-positive isolates can be enteropathogenic for humans. However, there may be differences in virulence between these subpopulations of *cpe*-positive *C. perfringens* isolates, based upon epidemiologic reports (5, 10, 17, 22, 26) indicating that the diarrheal symptoms of CPE-associated non-food-borne human GI diseases are generally more severe and of longer duration than the typical symptoms of *C. perfringens* type A food poisoning. Since the basis for these differences in symptomatology between CPE-associated human GI diseases remains unclear, future studies should compare the virulence phenotypes of chromosomal versus episomal *cpe*-positive isolates. Also, note that the identification of isolate 29 (which apparently carries a chromosomal *cpe* and which originated from a diarrheic pig) in the current study strongly suggests that both chromosomal (isolate 29) and episomal (13, 18; this study) *cpe*-positive isolates can be enteropathogenic for domestic animals.

Third, by implying that enterotoxigenic isolates from CPE-associated non-food-borne human GI diseases often (or always) differ genotypically from *C. perfringens* type A food-poisoning isolates, the current results also provide the first evidence that distinct subpopulations of *cpe*-positive *C. perfringens* isolates may be linked to particular types of CPE-associated human GI diseases. This insight could have potentially important diagnostic and epidemiologic implications. For example, if the associations suggested by this and previous genotyping studies are verified in further surveys, RFLP or PFGE assays for *cpe* would become useful for differentially diagnosing cases of CPE-associated food-borne versus non-food-borne diarrhea. In this regard, note that results with >20 isolates (13; this study) indicate that RFLP assays for *cpe* are very reliable predictors of whether an isolate carries an episomal versus chromosomal *cpe*. Therefore, RFLP appears to be a useful alternative for the presumptive identification of chromosomal versus episomal *cpe* isolates in laboratories lacking PFGE equipment (it also deserves mention that existing *cpe* detection-based assays other than PFGE and RFLP cannot distinguish chromosomal versus episomal *cpe*-positive isolates).

Similarly, *cpe* RFLP or PFGE analyses could prove to be useful for epidemiologic surveys for the identification of reservoirs for each genotypic subpopulation of *cpe*-positive isolates. Identification of these reservoirs, which are poorly understood at present, may allow for the introduction of measures specifically aimed at preventing the introduction of chromosomal *cpe* isolates into foods or preventing the spread of CPE-associated non-food-borne diseases, which may be exogenous infections (5, 7). These epidemiologic surveys should also better determine the relative prevalence of chromosomal versus episomal *cpe*-positive isolates, both globally and in specific ecologic niches. Although a previous study (18) claims that chromosomal *cpe* isolates are less common in nature than episomal *cpe* isolates, this conclusion appears premature since it was based upon surveys (13, 18) that predominantly examined veterinary isolates, which mostly carry an episomal *cpe* (13, 18; this study).

Finally, by strongly suggesting that many (or most) CPE-associated non-food-borne human GI disease isolates carry an episomal *cpe*, the current study also provides important additional support for the growing appreciation (18) of the important role that extrachromosomal genetic material plays in the virulence of clostridia involved in human infections. A future challenge will be to determine whether the *cpe*-carrying episome(s) present in most, or all, of our non-food-borne human GI disease (and most veterinary) isolates is a large plasmid or a phage capable of extrachromosomal replication.

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