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Genotypic and Phenotypic Characterization of *Clostridium perfringens* Isolates from Darmbrand Cases in Post-World War II Germany

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Clostridium perfringens **type C strains are the only non-type-A isolates that cause human disease. They are responsible for enteritis necroticans, which was termed Darmbrand when occurring in post-World War II Germany. Darmbrand strains were initially classified as type F because of their exceptional heat resistance but later identified as type C strains. Since only limited information exists regarding Darmbrand strains, this study genetically and phenotypically characterized seven 1940s era Darmbrand-associated strains. Results obtained indicated the following. (i) Five of these Darmbrand isolates belong to type C, carry beta-toxin (*cpb*) and enterotoxin (*cpe*) genes on large plasmids, and express both beta-toxin and enterotoxin. The other two isolates are *cpe*-negative type A. (ii) All seven isolates produce highly heat-resistant spores with D_{100} values (the time that a culture must be kept at 100°C to reduce its viability by 90%) of 7 to 40 min. (iii) All of the isolates surveyed produce the same variant small acid-soluble protein 4 (Ssp4) made by type A food poisoning isolates with a chromosomal *cpe* gene that also produce extremely heat-resistant spores. (iv) The Darmbrand isolates share a genetic background with type A chromosomal-*cpe*-bearing isolates. Finally, it was shown that both the *cpe* and *cpb* genes can be mobilized in Darmbrand isolates. These results suggest that *C. perfringens* type A and C strains that cause human food-borne illness share a spore heat resistance mechanism that likely favors their survival in temperature-abused food. They also suggest possible evolutionary relationships between Darmbrand strains and type A strains carrying a chromosomal *cpe* gene.**

Clostridium perfringens, an anaerobic, Gram-positive bacterium, exists ubiquitously in soils, sewage, food, and the normal intestinal flora of humans and animals (22). It is also a very important pathogen because of its ability to produce at least 16 different toxins, although individual isolates never express this entire toxin arsenal. On the basis of the production of four typing toxins (alpha, beta, epsilon, and iota), *C. perfringens* is classified into five types (A, B, C, D, and E). Each type is associated with different diseases that affect humans or animals (22). In livestock species, type C isolates cause fatal necrotizing enteritis and enterotoxemia (22). Type C strains are also the only non-type-A *C. perfringens* strains that cause human disease (22), which is referred to as enteritis necroticans, also known as pigbel or Darmbrand. Enteritis necroticans is a fatal disease that involves vomiting, severe abdominal pain, intestinal necrosis, and bloody stool (13, 14). Acute cases can result in rapid death.

By definition, type C isolates must produce alpha-toxin (CPA) and beta-toxin (CPB). CPB, a 35-kDa pore-forming polypeptide encoded by the plasmid-borne *cpb* gene, is necessary for type C strains to cause either necrotizing enteritis or enterotoxemia in animals (20, 31, 35–37). CPA is a 43-kDa protein with phospholipase C activity and the ability to activate endogenous signaling pathways in host cells (34). Besides CPA and CPB, some type C isolates produce additional toxins, such as the enterotoxin (CPE), which is encoded by a plasmid-borne *cpe* gene in all of the *cpe*-positive type C strains examined to date (11, 18). CPE, a 35-kDa pore-forming polypeptide expressed during sporulation, contributes to several important human and veterinary enteric diseases caused by type A strains (21). About 75 to 80% of all cases of *C. perfringens* type A food poisoning, which is the second most common bacterial food-borne illness in the United States (6, 32), are caused by type A strains carrying a chromosomal *cpe* gene (8, 9, 21, 26). In contrast, *cpe*-positive type A strains that cause human non-food-borne gastrointestinal diseases or animal diseases invariably carry the *cpe* gene on a large plasmid (8, 9, 18, 25, 38).

C. perfringens type A food poisoning isolates with a chromosomal *cpe* gene typically produce spores that are extremely resistant to standard food hygiene approaches such as heat, cold, or chemical preservatives (16, 29). For example, it was shown that spores made by type A chromosomal *cpe*-positive isolates exhibit, on average, a 60-fold higher decimal reduction value at 100°C (i.e., the D_{100} value, which is the time that a culture must be kept at 100°C to reduce its viability by 90%) than the less resistant spores produced by either type A isolates carrying a plasmid-borne *cpe* gene or non-type-A strains (17, 29). The exceptional heat resistance properties of spores made by most type A chromosomal *cpe*-positive strains is thought to favor their survival in temperature-abused foods (16, 17, 21, 29).

Alpha/beta-type small acid-soluble proteins (SASPs) protect spores from heat, cold, or sodium nitrite by binding to spore DNA (19, 28, 33). A variant of small, acid-soluble protein 4 (Ssp4) was recently identified as an important contributor to the exceptional resistance properties of the spores made by most type A chromosomal-*cpe*-carrying isolates (17, 19). Those chromosomal-*cpe*-carrying strains that produce highly heat-resistant spores make an Ssp4 variant with an Asp substitution at residue 36, instead of the Gly residue present at that position

Received 1 August 2012 **Returned for modification** 4 September 2012
Accepted 22 September 2012
Published ahead of print 1 October 2012
Editor: Steven R. Blanke
Address correspondence to Bruce McClane, bamcc@pitt.edu.

doi:10.1128/IAI.00818-12

TABLE 1 Isolates used in this study

Isolate	Type	Carriage of *cpb, cpe, pfoA, cpb2* genes
CN2076[a]	C	*cpb, cpe*
CN3748[a]	C	*cpb, cpe*
CN3752[a]	A	None
CN3753[a]	C	*cpb, cpe*
CN3758[a]	C	*cpb, cpe*
CN3763[a]	C	*cpb, cpe*
CN3765[a]	A	None
F5603[b]	A	*cpe, pfoA, cpb2*
NCTC10239[c]	A	*cpe*
CN5388[d]	C	*cpb, cpe*

[a] Darmbrand strains from the 1940s that were isolated by H. Zeissler, deposited in the Wellcome Collection, and later provided by R. Wilkinson.
[b] 1990s European sporadic-diarrhea isolate (2).
[c] 1950s food poisoning isolate (29).
[d] 1960s pigbel isolate (18).

TABLE 2 Primers used in this study

Primer	Sequence	Reference
gyrB-F	5′ ATTGTTGATAACAGTATTGATGAAGC	10
gyrB-R	5′ ATTTCCTAATTTAGTTTTAGTTTGC	
sigK-F	5′ CAATACTTATTAGAATTAGTTGGTAG	
sigK-R	5′ CTAGATACATATGATCTTGATATACC	
sod-F	5′ CAAAAAAAGTCCATTAATGTATCCAG	
sod-R	5′ TTATCTATTGTTATAATATTCTTCAC	
groEL-F	5′ TACAAGATTTATTACCATTACTTGAG	
groEL-R	5′ CATTTCTTTTTCTGGAATATCTGC	
pgk-F	5′ GACTTTAACGTTCCATTAAAAGATGG	
pgk-R	5′ CTAATCCCATGAATCCTTCAGCGATG	
nadA-F	5′ ATTAGCACATTATTATCAAATTCCTG	
nadA-R	5′ TTATATGCCTTTAATCTTAAATCCTC	
colA-F	5′ ATTAGAAAGTTTATGTACAATAGGTG	
colA-R2	5′ AAGACATTCTATTATTTCTATCGTAAGC	
plc-F	5′ AGGAACTCATGCTATGATTGTAACTC	
plc-R	5′ GGATCATTACCCTCTGATACATCGT	
Ssp4-F	5′ ATGAGCAAGACACCATTAAAAAA	17
Ssp4-R	5′ TTACTTTTCGTCAACGTGAGG	
cpbF	5′ GCAGGATCCATGAAGAAAAAATTTAT	This study
cpbR	5′ ATACTCGAGCTAAATAGCTGTTACTTT	
cpeF	5′ AAAGGAGATGGTTGGATATTAGG	This study
cpeR	5′ GTCCAAGGGTATGAGTTAGAAG	
pfoA-F	5′ TTTATGAACTTAACAAATGAGGGG	This study
pfoA-R	5′ CTACTCCAAGTGAGTTTTCAAGG	
cpb2-F	5′ AGATTTTAAATATGATCCTAACC	
cpb2-R	5′ CAATACCCTTCACCAAATACTC	
F1	5′ TCTAGTTACCCTAGAAAGCATTACT	11
R1	5′ GGAAGGTCCTCACTTATCAT	
F2	5′ ATGATAAGTGAGGACCTTCC	
R2	5′ GCTCTAAAAAAGAGCTTAAAAGCA	
F3	5′ CTGCTTTTAAGCTCTTTTTTAGAGC	
R3	5′ TGGTCATATTTCATGTATAACT	
F4	5′ AGTTATACATGAAATATGACCA	
R4	5′ CCTCCTTTTGTATATAGATGATCTG	
F5	5′ CAGATCATCTATATACAAAAGGAGG	
R5	5′ CCAGTTAACACCATTCCAATTAAGA	
TnF	5′ ATACATTAACTAACTTAGAACGTAC	30
BetaR	5′ GAAAGAAACTGTTATTATCTTAATTG	
dcmRseq	5′ TCACCCAACAAGTAACTATAATG	18
cpeMR	5′ TTAGAACAGTCCTTAGGTGATGGA	

in the Ssp4 variant made by *C. perfringens* strains that produce more heat-sensitive spores (17, 19).

Darmbrand was a severe human illness that occurred in the malnourished people of northern Germany from 1944 to 1949 (14, 39). The disease was often fatal because of necrotic inflammation of the small intestine, especially in the jejunum. *Clostridium welchii*, now named *C. perfringens*, was identified as the causative bacterium. Because *C. welchii* Darmbrand isolates produced unusually heat-resistant spores that could resist boiling for 1 to 4 h, they were initially classified as *C. welchii* type F (39). For example, one reported Darmbrand case occurred in a person who ate canned rabbit meat that had been boiled for 2 h (39). However, when the production of CPB by Darmbrand isolates was later detected using beta-antitoxin (14, 39), these type F isolates were reclassified as type C strains.

Darmbrand isolates have been little studied since the 1940s and 1950s, so the present investigation applied modern techniques to characterize several Darmbrand strains genotypically and phenotypically and compare them with other *C. perfringens* human enteric disease strains.

MATERIALS AND METHODS

Bacteria, media, and reagents. The *C. perfringens* isolates examined in this study, including the 1940s Darmbrand strains, are listed and described in Table 1. Carriage of *cpb*, *cpe*, *pfoA*, and *cpb2* genes by these isolates was evaluated by PCR as described below. All isolates were stored as cooked meat medium stock cultures at −20°C in our lab. Fluid thioglycolate (FTG) medium (Difco Laboratories) and TGY medium (3% tryptic soy broth [BD], 2% glucose [Fisher Scientific], 1% yeast extract [BD], 0.1% sodium thioglycolate [Sigma-Aldrich]) were used for broth cultures. Brain heart infusion (BHI; Difco Laboratories) agar plates were used for bacterial colony counting, and LB broth (10% tryptone [BD], 5% yeast extract [BD], 5% sodium chloride [Fisher Scientific]) was used to grow *Escherichia coli*. Modified Duncan-Strong (MDS) medium (7) was used to induce the sporulation of all *C. perfringens* isolates and CPE production by the *cpe*-positive strains.

PCR analyses of carriage of *cpe*, *cpb*, *pfoA*, and *cpb2* genes. Template DNA for PCRs was extracted from overnight TGY medium cultures and then purified using the MasterPure Gram-positive DNA purification kit (Epicentre, Madison, WI). Each PCR mixture (20 μl) contained 2 μl of template DNA, 10 μl of *Taq* complete 2× Master Mix (New England BioLabs), and 1 μl of each primer pair (1 μM final concentration). The primers used in this investigation are listed in Table 2. The amplification conditions used for these PCR analyses were 1 cycle of 94°C for 2 min and 35 cycles of 94°C for 30 s, 55°C for 40 s, and 68°C for 40 s, followed by a final single extension of 8 min at 68°C. The PCR products were electrophoresed on a 1.5% agarose gel and stained with ethidium bromide for visualization.

PCR analysis and sequencing of the *ssp4* gene. PCR was performed to detect the presence of the *ssp4* gene in Darmbrand isolates using purified DNA, prepared as described above, and specific primers listed in Table 2. The PCR conditions were 1 cycle of 94°C for 2 min and 35 cycles of 94°C for 30 s, 52°C for 30 s, and 68°C for 30 s, followed by a final single extension of 8 min at 68°C. The *ssp4* PCR products were electrophoresed on a 1.5% agarose gel and stained with ethidium bromide for visualization. For sequencing analyses, the PCR products were cloned into the pCR2.1-TOPO vector (Invitrogen) and then transformed into *E. coli* DH5α competent cells (Invitrogen), which were then cultured overnight on BHI agar

plates containing 50 μg/ml kanamycin at 37°C. Single colonies were subcultured in LB broth overnight at 37°C, and the plasmid was then extracted with a QIAprep Spin Miniprep kit (Qiagen). The PCR products in the plasmid were then sequenced at the University of Pittsburgh Core DNA Sequencing Facility.

Southern blot analyses. Genomic *C. perfringens* DNA was purified using the MasterPure Gram-positive DNA purification kit (Epicentre, Madison, WI). Each purified DNA sample (2.5 μg) was digested overnight with XbaI at 37°C according to the manufacturer's (New England BioLabs) instructions. The digested DNA samples were electrophoresed on a 1% agarose gel, and the separated DNA digestion products were then transferred onto positively charged nylon membranes (Roche) for hybridization with either a *cpe* or a *cpb* probe. Digoxigenin (DIG)-labeled *cpe* or *cpb* probes were prepared using a PCR DIG probe synthesis kit (Roche); the primers used to make the *cpb* and *cpe* probes are listed in Table 2. After hybridization with these probes, the Southern blot was developed by using reagents from the DIG DNA labeling and detection kit (Roche) according to the manufacturer's instructions.

Pulsed-field gel electrophoresis (PFGE). Individual *C. perfringens* isolates were grown overnight in 10 ml of FTG broth at 37°C. A 0.1-ml aliquot of each overnight culture was then inoculated into 10 ml of TGY medium. After overnight growth at 37°C, the TGY medium cultures were collected to prepare genomic DNA agarose plugs. Bacterial cells were harvested by centrifugation at 9,000 × *g*, washed three times with TES buffer (1 M Tris, 0.5 M EDTA, 5 M sucrose [pH 8.0]), resuspended in 200 μl of TE buffer, and then embedded in 200 μl of melted 2% certified low-melting-point agarose (Bio-Rad Laboratories). Plugs were prepared and cut into 2- to 3-mm slices. The agarose-embedded bacterial cells were lysed overnight at 37°C in lysis buffer (500 μM EDTA [pH 8.0], 0.5% Sarkosyl, 0.5% lysozyme [Sigma], 0.4% deoxycholic acid) with gentle shaking of the plugs, followed by a 2-day incubation at 55°C in 0.2% proteinase K (Gene Choice)–500 μM EDTA (pH 8.0) buffer. PFGE of these samples was then performed by using a 1% agarose gel in 0.5× TBE buffer at 14°C and the CHEF-DR II system (Bio-Rad Laboratories). The gel running parameters, as described previously (11, 18), were as follows: initial pulse, 1 s; final pulse, 25 s; voltage, 6 V/cm; time, 24 h.

Southern blot analyses of pulsed-field gels. The DIG-labeled DNA probes used for pulsed-field Southern blot analyses were the same *cpe* and *cpb* probes described above for Southern blot assays of regular agarose gels. Southern hybridization of pulsed-field gels was performed as described previously (30). CSPD substrate (Roche) was used for detection of the Southern blot-hybridized bands according to the manufacturer's protocol.

Western blot analyses of CPB and CPE production by Darmbrand isolates. Isolates were grown overnight in FTG medium at 37°C. An aliquot (100 μl) of each culture was then inoculated into 10 ml of TGY medium (for CPB production) or 10 ml of fresh MDS medium (for CPE production). After overnight incubation at 37°C, cultures were centrifuged at 8,000 × *g* for 5 min and the same volume of each culture supernatant was mixed with SDS-PAGE loading buffer and boiled for 5 min. The samples were electrophoresed on a 12% polyacrylamide gel containing SDS, and separated proteins were then transferred onto nitrocellulose membranes. The membranes were blocked with Tris-buffered saline–Tween 20 (0.05%, vol/vol) and nonfat dry milk (5%, wt/vol) for 1 h before each membrane was probed with either rabbit anti-CPE polyclonal antiserum (15) or mouse anti-CPB monoclonal antibody (20, 31). Bound antibody was then detected after incubation with a horseradish peroxidase-conjugated, species-specific antibody and addition of SuperSignal West Pico Chemiluminescent Substrate (Pierce).

Measurement of spore heat resistance. The moist heat resistance of spores made by Darmbrand isolates was determined by calculating their D_{100} values (17, 29). For this purpose, an aliquot (0.1 ml) of an overnight FTG medium culture of each isolate was inoculated into 10 ml of fresh MDS medium (7) and incubated overnight at 37°C. The overnight culture was then heat shocked for 20 min at 70°C to kill the vegetative cells. After covering the cells and boiling them for 0 to 60 min, each heat-shocked culture was 10-fold serially diluted from 10^{-2} to 10^{-7} with sterile water and plated onto BHI agar plates for colony counting. The plates were incubated overnight under anaerobic conditions. Each experiment was separately repeated three times.

MLST analysis. To investigate the genetic relatedness of the Darmbrand isolates with other *C. perfringens* strains, multilocus sequencing typing (MLST) analysis of the Darmbrand strains was performed by sequencing eight housekeeping genes. As described previously for other *C. perfringens* strains (10), these genes included *plc* (encodes alpha-toxin), *colA* (encodes collagenase A), *sodA* (superoxide dismutase gene), *groEL* (heat shock protein gene), *sigK* (encodes sigma factor K), *pgk* and *nadA* (encode putative metabolism genes), and *gyrB* (DNA gyrase B gene). The primers used to amplify these eight housekeeping genes are listed in Table 2. Each PCR mixture (50 μl) contained 5 μl of template DNA, 25 μl of *Taq* complete 2× Master Mix (New England BioLabs), 2.5 μl of each primer pair (1 μM final concentration), and 15 μl of PCR grade water. PCRs for all eight housekeeping gene amplifications were performed under the same conditions: 1 cycle of 94°C for 2 min and 35 cycles of 94°C for 30 s, 55°C for 60 s, and 68°C for 60 s, followed by a final single extension of 10 min at 68°C. The PCR products were electrophoresed on a 1.5% agarose gel and stained with ethidium bromide for visualization. PCR products were purified with a QIAquick PCR purification kit (GIAGEN) and then sequenced at the University of Pittsburgh Core DNA Sequencing Facility. All sequence data were concatenated to produce an in-frame 5,274-bp nucleotide sequence according to the genome arrangement of each gene in strain 13. Sequence information for these eight housekeeping genes in three reference strains, whose whole genomes have been completely sequenced and assembled, was also used in this analysis. Concatenated sequence data were applied to phylogenetic analysis in the ClustalW format using Winxyz software.

Overlapping PCR and long-range PCR analyses to evaluate *cpb* locus organization in type C Darmbrand isolates. Long-range and overlapping PCR analyses were performed to evaluate the diversity of the *cpb* locus among the five Darmbrand isolates identified as type C (see Results). These analyses used the primers listed in Table 2 and template DNA purified using the MasterPure Gram-positive DNA purification kit. Each PCR mixture contained 5 μl of template DNA, 25 μl of *Taq* 2× Master Mix (New England BioLabs), 1 μl of forward primer, 1 μl of reverse primer (1 μM final concentration), and 20 μl of distilled water. Long-range PCRs were performed with a Techne thermocycler using the following PCR conditions for the amplifications: 95°C for 2 min and 35 cycles of 94°C for 30 s, 54°C for 30 s, and 65°C for 3 min, followed by a final single extension of 10 min at 65°C. Overlapping PCRs were performed under the following amplification conditions: 35 cycles of 94°C for 30 s, 54°C for 30 s, and 68°C for 1 min, followed by a final single extension of 10 min at 68°C. PCR products were separated on a 1.5% agarose gel and visualized by ethidium bromide staining. PCR products were then purified with the QIAquick PCR purification kit (Qiagen) and sequenced at the University of Pittsburgh Core DNA Sequencing Facility.

To study sequences downstream of the *cpb* gene in Darmbrand strains, plasmid DNA was extracted from fresh overnight TGY medium cultures using the QIAprep Spin Miniprep kit (Qiagen). The extracted plasmids were then used as templates for sequencing downstream of the *cpb* gene.

PCR identification of possible circular transposition intermediates carrying the *cpb* gene. As indicated above, genomic *C. perfringens* DNA was freshly extracted from BHI agar cultures grown anaerobically overnight. Each PCR mixture contained 5 μl of template DNA, 25 μl of *Taq* 2× Master Mix (New England BioLabs), 1 μl each of primers TnF and betaR (1 μM final concentration), and 20 μl of distilled water. PCR amplification was then performed with a Techne thermocycler under the following conditions: 95°C for 2 min and 35 cycles of 94°C for 30 s, 54°C for 30 s, and 68°C for 1.5 min, followed by a final single extension of 7 min at 68°C. PCR products were separated on a 1.5% agarose gel and visualized by ethidium bromide staining. PCR products were then excised from the

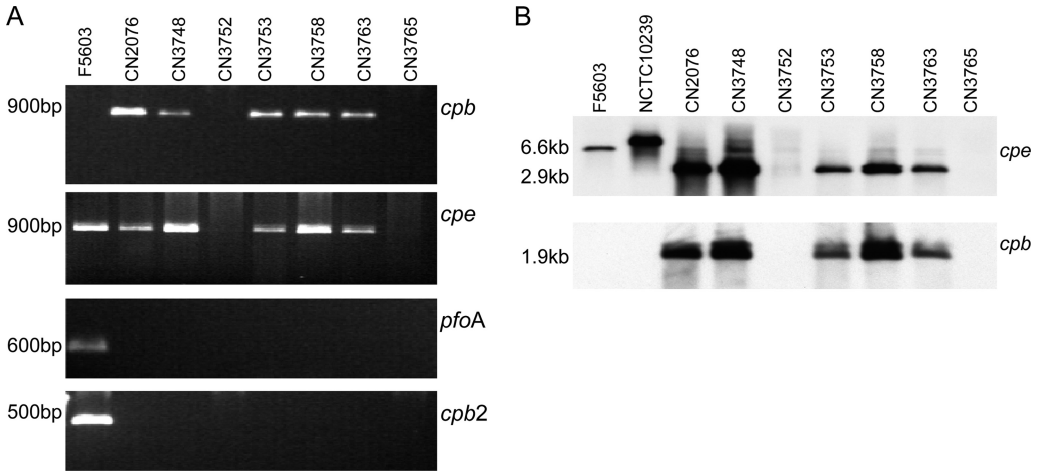

FIG 1 PCR assay and Southern blot analysis. (A) PCR analysis of *cpb*, *cpe*, *pfoA*, and *cpb2* gene carriage in the seven Darmbrand isolates surveyed. Type A *cpe*-positive isolate F5603 was also included as a positive control for detection of the *cpe*, *cpb2*, and *pfoA* genes (10, 25). Expected sizes of PCR products are indicated on the left. (B) Southern blot hybridization. The genomic DNA of each isolate was digested with XbaI overnight at 37°C and then subjected to 1% agarose electrophoresis prior to Southern hybridization with a DIG-labeled *cpe* or *cpb* probe. Type A *cpe*-positive isolates F5603 and NCTC10239 were used as positive controls for the *cpe* gene. The sizes of detected bands are shown on the left side of the blots.

gel using a Quantum Prep Freeze N Squeeze DNA gel extraction spin column (Bio-Rad) and sequenced at the University of Pittsburgh Core DNA Sequencing Facility.

PCR identification of potential *cpe* gene mobilization. Each PCR mixture contained 5 μl of template DNA, which was freshly extracted from single colonies growing overnight on anaerobically cultured BHI agar plates, 25 μl of *Taq* 2× Master Mix (New England BioLabs), 1 μl each of primers dcmRseq and cpeMR (1 μM final concentration), and 20 μl of distilled water. PCR amplification was then performed with a Techne thermocycler under the following conditions: 95°C for 2 min and 35 cycles of 94°C for 30 s, 54°C for 30 s, and 68°C for 2 min, followed by a final single extension of 10 min at 68°C. PCR products were separated on 1.5% agarose gels and visualized by ethidium bromide staining. PCR products were then purified with the QIAquick PCR purification kit (Qiagen) and sequenced at the University of Pittsburgh Core DNA Sequencing Facility.

Statistical analyses. Statistical analyses were performed using the Student *t* test.

Nucleotide sequence accession numbers. The sequences determined in this investigation have been deposited in the GenBank database under accession numbers JX267142 to JX267169, JX276507 to JX276534, and JX308274 to JX308280.

RESULTS

PCR analysis of *cpb*, *cpe*, *pfoA*, and *cpb2* gene carriage by Darmbrand isolates. Using DNA prepared from Darmbrand strains CN2076, CN3748, CN3753, CN3758, and CN3763, PCR assays amplified products of the expected size indicating carriage of the *cpb* and *cpe* genes (Fig. 1A). However, DNA from Darmbrand strains CN3752 and CN3765 (Fig. 1A) did not support PCR amplification of either *cpb* or *cpe* products. In addition, these PCR assays revealed that none of these seven Darmbrand strains carry the *pfoA* or *cpb2* gene (Fig. 1A). Consistent with their lack of the *pfoA* gene, the seven Darmbrand isolates did not produce the typical perfringolysin O-induced inner zone of beta hemolysis that develops when most *C. perfringens* strains are grown on sheep blood agar plates (data not shown).

Southern blot assays using *cpe* and *cpb* probes confirmed the carriage of the *cpe* and *cpb* toxin genes by the five Darmbrand isolates that had tested positive by PCR for the possession of those two toxin genes (Fig. 1B). Thus, those five strains genotypically belong to type C, while Darmbrand strains CN3752 and CN3765 are classified genotypically as *cpe*-negative type A (Table 1).

Pulsed-field gel Southern blot analysis to investigate *cpb* and *cpe* gene locations in Darmbrand isolates. PFGE conditions have been established that allow *C. perfringens* plasmid, but not chromosomal, DNA to enter a pulsed-field gel and migrate according to its molecular size (11, 18, 25, 30). Previous studies using Southern blot analyses of similar pulsed-field gels demonstrated that most type A food poisoning isolates carry a chromosomal *cpe* gene, while *cpe*-positive type A non-food-borne disease isolates and type C animal disease isolates carry the *cpe* gene on large plasmids (7–9, 18, 26).

Therefore, the collection of Darmbrand isolates was subjected to a similar PFGE analysis, followed by sequential Southern hybridization with a *cpe*-specific probe. As shown in Fig. 2, these

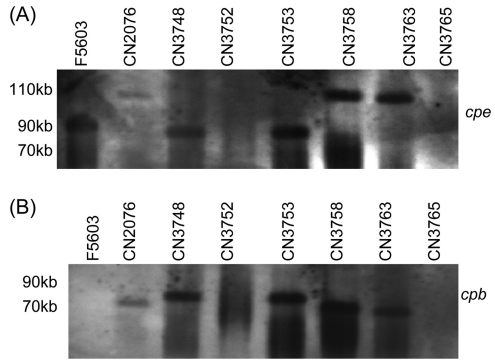

FIG 2 PFGE and Southern blot analysis of the seven Darmbrand isolates surveyed. Type A isolate F5603 was included as a positive control for a type A plasmid *cpe*-positive strain. (A) Genomic DNAs from all of the strains were subjected to PFGE prior to Southern blot hybridization with a DIG-labeled *cpe*-specific probe. (B) The same blot was stripped and then reprobed with a DIG-labeled *cpb*-specific probe. The migration of molecular size markers is indicated on the left side of the blots.

FIG 3 Western blot analysis of CPE and CPB production by Darmbrand isolates CN2076, CN3748, CN3752, CN3753, CN3758, CN3763, and CN3765. Type A isolate F5603 was used as a positive control for CPE production. (A) Detection of the production of CPE using a CPE-specific polyclonal antibody. The bacteria were cultured in MDS medium overnight at 37°C, and culture supernatants were then subjected to CPE Western blotting. The migration of purified CPE (35 kDa) is shown in the right lane of the blot. (B) Detection of CPB production. The bacteria were grown in TGY broth overnight at 37°C, and culture supernatants were then subjected to Western blotting using a CPB-specific monoclonal antibody. The migration of purified CPB (35 kDa) is shown in the right lane of the blot.

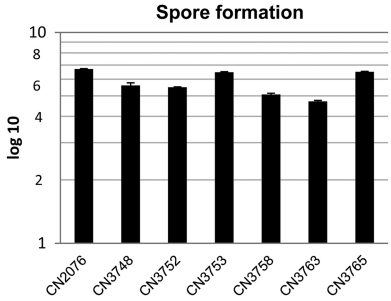

FIG 4 Spore formation by Darmbrand strains CN2076, CN3748, CN3752, CN3753, CN3758, CN3763, and CN3765. The bacteria were grown in MDS medium overnight at 37°C for sporulation, and the overnight cultures were then heat shocked for 20 min at 70°C. After 10-fold serial dilution with distilled water, the bacteria were plated onto BHI agar plates and grown overnight at 37°C for colony counting. Shown are mean results ($\log_{10}$ scale) from three independent repetitions. Error bars depict standard deviations.

analyses showed that the five *cpe*-positive type C Darmbrand isolates surveyed carry the *cpe* gene on large plasmids, with a size of either ~75 or ~110 kb (Fig. 2A). It was notable that Darmbrand strain CN3758 was determined to carry an ~110-kb *cpe*-bearing plasmid in this study since our laboratory previously reported (18) that the *cpe*-bearing plasmid in this strain is only ~75 kb. However, when several stock cultures of this strain were rechecked during the present work, the *cpe*-bearing plasmid consistently ran with a size of ~110 kb, suggesting possible mislabeling of the strain during the previous study (18).

The same Southern blots were then stripped of the *cpe* probe and rehybridized with a *cpb* probe. Results from these analyses showed that, compared to their *cpe*-bearing plasmids, the *cpb*-bearing plasmids in these five type C Darmbrand strains are more diverse (Fig. 2B), with sizes ranging from ~65 to 85 kb. Overlaying of the Fig. 2A and B blots confirmed (data not shown) that the *cpb* and *cpe* genes are present on two distinct plasmids in Darmbrand strains CN2076, CN3758, and CN3765. However, the *cpb* and *cpe* probes hybridized to the same location in the Southern blot assays using DNA from CN3748 and CN3753, which could indicate that the *cpb* and *cpe* genes in these two isolates are present on either (i) the same 85-kb plasmid or (ii) two different plasmids that comigrate on the pulsed-field gel because of their similarity in size.

Production of CPB and CPE by Darmbrand strains. The expression of CPE and CPB by the Darmbrand isolates surveyed was evaluated by Western blot analysis. Using a CPE-specific antibody, CPE production was detected in sporulating cultures of isolates CN2076, CN3748, CN3753, CN3758, and CN3763 but not isolates CN3752 and CN3765 (Fig. 3A); those results are in agreement with the Fig. 1 PCR and Southern blot results indicating that the last two Darmbrand strains do not carry a *cpe* gene. Using a CPB-specific monoclonal antibody, CPB production was also detected in overnight TGY medium cultures of Darmbrand isolates CN2076, CN3748, CN3753, CN3758, and CN3763 but not in those of CN3752 and CN3765 (Fig. 3B), which also matches the Fig. 1 PCR and Southern blot results indicating that the last two Darmbrand strains do not carry a *cpb* gene.

Darmbrand strain spore heat resistance. The ability of the Darmbrand isolates surveyed to sporulate was first assessed by colony counting after MDS medium cultures had been heat shocked for 20 min at 70°C. Those analyses revealed approximately similar levels of sporulation by all seven Darmbrand isolates under these culture conditions (Fig. 4).

Therefore, the heat resistance properties of spores of these Darmbrand isolates were assessed by boiling each culture for various times. Except for strain CN3765, which withstood boiling for up to 15 min, viable spores were recovered from all of the Darmbrand isolate cultures after boiling for 60 min (data not shown). When a D_{100} value (the time at 100°C needed to reduce spore viability by 1 $\log_{10}$) was calculated for spores of each Darmbrand isolate, the values determined ranged from 7 to 40 min (Table 3). For comparison, these values are significantly longer than the <1-min and ~2.4-min D_{100} values calculated (Table 3), respectively, for *C. perfringens* strains F5603 and CN5388, which are (respectively) a type A strain carrying a *cpe*-bearing plasmid (2, 8) and a *cpe*-positive type C pigbel strain (19).

Sequencing of the *ssp4* gene of Darmbrand isolates. A variant small acid-soluble protein 4, which is encoded by the *ssp4* gene, was recently demonstrated to be important for the exceptional resistance properties of spores made by most type A isolates carrying a chromosomal *cpe* gene (17). For that Ssp4 variant, the Asp located at residue 36 was shown to play a critical role when this

TABLE 3 Spore heat (100°C) resistance

Strain	Type	Mean D_{100} value (min) ± SD
CN2076	C	13.5 ± 0.5[c]
CN3748	C	16.6 ± 0.6[c]
CN3752	A	40.3 ± 0.8[c]
CN3753	C	20.8 ± 1.0[c]
CN3758	C	20.5 ± 1.3[c]
CN3763	C	33.4 ± 0.7[c]
CN3765	A	7.0 ± 0.5[c]
F4969[a]	A	0.5 ± 0.0
F5603	A	0.9 ± 0.3
F5603[b]	A	0.6
CN5388	C	2.4 ± 0.4
CN5388[a]	C	2.3 ± 0.3
SM101[a]	A	59.1 ± 1.3

[a] Data from reference 17 for comparison.
[b] Data from reference 29 for comparison.
[c] $P < 0.0001$ compared with strain CN5388.

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F4969   MSKTPLKKIIKGKIKSNKELTPAEKLREKMKYEIAGELGLSDKVDKFGWGGLTAEETGRIGGLMTKRKKELKLPSNDEILGRKKPHVDEK
SM101   MSKTPLKKIIKGKIKSNKELTPAEELREKMKYEIADELGLSDKVDKFGWGGLTAEETGRIGGLMTKRKKELNLPSNDEILGRKKPHVDEK
CN2076  MSKTPLKKIIKGKIKSNKELTPAEKLREKMKYEIADELGLSDKVDKFGWGGLTAEETGRIGGLMTKRKKELNLPSNDEILGRKKPHVDEK
CN3748  MSKTPLKKIIKGKIKSNKELTPAEKLREKMKYEIADELGLSDKVDKFGWGGLTAEETGRIGGLMTKRKKELNLPSNDEILGRKKPHVDEK
CN3752  MSKTPLKKIIKGKIKSNKELTPAEKLREKMKYEIADELGLSDKVDKFGWGGLTAEETGRIGGLMTKRKKELNLPSNDEILGRKKPHVDEK
CN3753  MSKTPLKKIIKGKIKSNKELTPAEKLREKMKYEIADELGLSDKVDKFGWGGLTAEETGRIGGLMTKRKKELNLPSNDEILGRKKPHVDEK
CN3758  MSKTPLKKIIKGKIKSNKELTPAEKLREKMKYEIADELGLSDKVDKFGWGGLTAEETGRIGGLMTKRKKELNLPSNDEILGRKKPHVDEK
CN3763  MSKTPLKKIIKGKIKSNKELTPAEKLREKMKYEIADELGLSDKVDKFGWGGLTAEETGRIGGLMTKRKKELNLPSNDEILGRKKPHVDEK
CN3765  MSKTPLKKIIKGKIKSNKELTPAEKLREKMKYEIADELGLSDKVDKFGWGGLTAEETGRIGGLMTKRKKELKLPSNDEILGRKKPHVDEK
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FIG 5 Deduced amino acid sequences encoded by the *ssp4* ORF of Darmbrand strains CN2076, CN3748, CN3752, CN3753, CN3758, CN3763, and CN3765. Deduced Ssp4 sequences of type A strains F4969 and SM101, which produce (respectively) heat-sensitive spores and extremely heat-resistant spores (29) were used for comparison here. Instead of the Gly (G) residue present at position 36 in strain F4969, which forms heat-sensitive spores (19), Darmbrand strains encode an Ssp4 protein with an Asp (D) residue substitution at position 36, which was shown to increase the heat resistance of strain SM101 spores (17).

protein mediates strong spore heat resistance; in contrast, *C. perfringens* strains that produce more heat-sensitive spores have a Gly residue present at Ssp4 position 36 (17).

To start assessing the basis for the relatively strong heat resistance of spores of Darmbrand strains shown in Table 3, the *ssp4* open reading frames (ORFs) of the seven Darmbrand isolates surveyed were sequenced. Interestingly, the *ssp4* gene of all seven Darmbrand isolates was found to encode an Asp at residue 36 of Ssp4 (Fig. 5).

MLST analysis of the seven Darmbrand isolates. Recent MLST studies indicated that chromosomal-*cpe*-bearing isolates share a genetic background and belong to a distinct cluster on the *C. perfringens* phylogenetic tree (10). Those MLST analyses also revealed that type A strains carrying a plasmid with *cpe* are somewhat related to one another but distinct from the type A chromosomal *cpe*-positive strains. Therefore, using the same eight representative housekeeping genes sequenced in that previous investigation, MLST analysis of the seven Darmbrand isolates surveyed was performed in order to find out whether those isolates are similar to one another and to determine if they resemble type A chromosomal *cpe*-positive strains or type A or C plasmid *cpe*-positive strains.

Results from the present MLST analyses indicated that these Darmbrand isolates share a genetic background with each other (Fig. 6). Furthermore, although these strains carry a plasmid-borne *cpe* gene, their genetic background resembles that of the previously characterized type A food poisoning chromosomal *cpe*-positive strains, as well as one type C Darmbrand strain (NCTC8081) not included in the present survey (10). All of the type A food poisoning chromosomal *cpe*-positive strains and the Darmbrand isolates, which produce heat-resistant spores, assembled into one definitive phylogenetic cluster (cluster I). However, all type A plasmid *cpe*-positive strains, type A *cpe*-negative isolates, and type C plasmid *cpe*-positive animal isolates assembled into a different cluster (cluster II).

Overlapping PCR analyses of *cpb* locus organization in Darmbrand strains. Previous studies in our lab had identified *dcm*, two IS*1151*-like sequences, and an IS*cpe7* sequence upstream of the *cpb* gene in type C strains JGS1495 and CN5388 (11), which do not have a Darmbrand origin. Therefore, overlapping PCR assays were performed to evaluate whether similar sequences are present upstream of the *cpb* gene in Darmbrand type C strains CN2076, CN3748, CN3753, CN3758, and CN3763. Five sets of primers (Table 2), amplifying PCR products A to E, showed the presence of IS*1151*-like sequence in all five isolates. The *dcm* gene was amplified from four Darmbrand type C isolates (CN2076, CN3753, CN3758, and CN3763) (Fig. 7A), but overlapping PCRs did not suggest the presence of these *dcm* genes near IS*1151*-like sequences or the *cpb* gene. Therefore, a long-range PCR analysis using primer sets F1/R5, F1/R3, and F3/R5 was performed. Long-range PCR with primer set F1/R3 and/or F1/R5 gave no PCR amplification (data not shown), which implied that the *dcm* genes are not adjacent to IS*1151*-like sequences or to the *cpb* gene in these isolates. PCR with F3/R5 suggested that IS*1151*-like sequences are located near the *cpb* gene in four of these isolates, i.e., CN2076, CN3748, CN37553, and CN3758, but not in CN3763 (Fig. 7B).

Consistent with those PCR results, sequencing of the long-range PCR products generated with primers F3 and R5 showed that four Darmbrand strains carry a novel *cpb* locus that differs from the previously described *cpb* loci of type B strain ATCC 3626, type C veterinary strain JGS1495, or type C human pigbel strain CN5388 (11, 30). Specifically, this sequencing showed that, in those four Darmbrand isolates, two IS*1151*-like sequences, one IS*1470* element, a Tn*C1* sequence, and IS*cpe7* are present upstream of the *cpb* gene, while an IS*cpe5* sequence is located downstream of the *cpb* gene (Fig. 7C).

PCR identification of possible circular transposition intermediates containing the *cpb* gene. The results shown in Fig. 7C indicated that, when present in the Darmbrand strains surveyed, the *cpb* gene is associated with insertion sequences that might mobilize that toxin gene. Supporting this possibility is the fact that *cpb*-containing circular transposition intermediates have been detected in other *C. perfringens* strains (30).

Therefore, a reverse PCR was performed with primers in opposite orientations to evaluate whether similar *cpb*-containing circular intermediates might also be formed in the Darmbrand isolates surveyed. Primers TnF and BetaR amplified a 1.5-kb PCR product from isolates CN3753, CN3758, and CN3763 (Fig. 7D). Those PCR products were purified and sequenced, which confirmed the amplification of *cpb*, IS*1470*, IS*1151*, and IS*cpe5* sequences (Fig. 7E).

PCR identification of a possible circular transposition containing the *cpe* gene. The sequence results shown in Fig. 8A indicated that the *cpe* gene is associated with insertion sequences that might mobilize the *cpe* gene. Supporting that possibility is the fact that potential *cpe*-containing circular transposition intermediates have been detected in some other *C. perfringens* strains (3, 18).

Consequently, reverse PCRs were performed by using primers in opposite orientations to evaluate whether similar *cpe*-containing circular intermediates are formed in the *cpe*-positive Darmbrand isolates surveyed. Primers dcmRseq and cpeMR consistently amplified a 1.6-kb PCR product from the five *cpe*-positive Darmbrand isolates, i.e., CN2076, CN3748, CN3753, CN3758, and CN3763 (Fig. 8B). Those PCR products were purified and

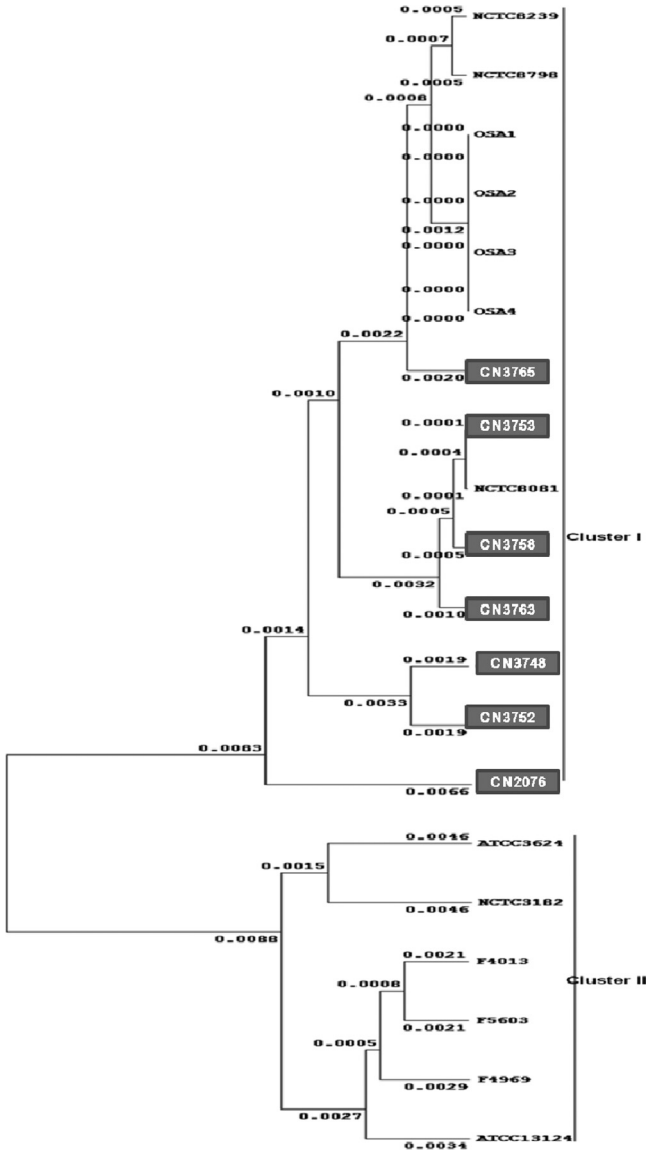

FIG 6 Phylogenetic relationships among 20 *C. perfringens* strains. Included are six previously genotyped (10) type A human food poisoning isolates carrying a chromosomal *cpe* gene (i.e., NCTC8239, NCTC8798, OSAKA1, OSAKA2, OSAKA3, and OSAKA4), the seven newly characterized Darmbrand isolates from this study (boxed), one previously examined (10) type C Darmbrand strain (NCTC8081), three previously genotyped (10) type A human sporadic-diarrhea isolates carrying a plasmid-borne *cpe* gene (F5603, F4969, and F4013), one *cpe*-negative type A strain ATCC 13124 (10), and one *cpe*-negative type C strain (NCTC3182) isolated from a diseased sheep (10). The phylogenetic tree was constructed by ClustalW format analysis using Winxyz software based on the concatenated 5,274-bp nucleotide sequence of eight housekeeping genes.

sequenced, which confirmed the amplification of a circular intermediate containing *cpe* sequences, an intact IS*1470* sequence, and a partial IS*1470* sequence (Fig. 8C), similar to results obtained previously with another Darmbrand isolate, i.e., CN2078 (18).

DISCUSSION

Food-borne illnesses, which remain a major public health problem, often involve foods that were cooked or stored at suboptimal temperatures. Therefore, it should be advantageous for *C. perfringens* strains that cause human food-borne illness to acquire an enhanced ability to survive in those temperature-abused foods (21). Consistent with this, ~75 to 80% of *C. perfringens* type A food poisoning outbreaks involve chromosomal-*cpe*-carrying type A strains whose spores exhibit exceptional temperature resistance properties (21, 26, 29). Specifically, the spores made by most chromosomal *cpe*-positive type A strains possess D_{100} values ranging from 30 to 120 min, in contrast to the 0.5- to 2.7-min D_{100} values of spores made by other type A strains or most non-type-A strains (17, 29).

Many years ago, Darmbrand strains were also identified as possessing unusually strong heat resistance properties (39). Specifically, their spores were reported to survive boiling, which our results confirmed. However, to our knowledge, the D_{100} value of spores made by Darmbrand strains has not been determined, which precluded direct quantitative comparisons of the resistance properties of spores made by Darmbrand strains with those of spores from other *C. perfringens* strains. In response, the present investigation calculated that the D_{100} values of spores made by seven Darmbrand strains ranged from 7 to 40 min, which is near the range of D_{100} values determined for spores of most chromosomal *cpe*-positive type A strains and substantially greater than the D_{100} value ranges calculated for spores made by other *C. perfringens* strains. Therefore, these and previous data (17, 29) establish that two *C. perfringens* food-borne diseases of humans, i.e., *C. perfringens* type A food poisoning and Darmbrand, often involve strains that produce highly heat-resistant spores, which likely favors their survival in temperature-abused foods.

Recent studies determined that the exceptional heat resistance properties of spores typically made by type A strains with a chromosomal *cpe* gene is attributable, in large part, to their production of a variant Ssp4 protein (17). In this Ssp4 variant, an Asp (rather than a Gly) residue is present at position 36 (note that Ssp4 proteins can also vary at residue 72, but this second variation does not affect spore heat resistance properties [17]). Subsequent studies (19) showed that the Asp36 Ssp4 variant binds more tightly than the Gly36 Ssp4 variant to *C. perfringens* DNA, which presumably provides spore DNA with greater protection against stresses such as heat. Therefore, our present determination that Darmbrand isolates also carry an *ssp4* gene that encodes the Ssp4 variant with the Asp36 residue strongly suggests a mechanistic basis for the strong heat resistance properties exhibited by spores made by Darmbrand strains. It is notable in this regard that, to date, the only identified *C. perfringens* isolates that encode the Ssp4 Asp36 variant are associated with food-borne human diseases, i.e., either *C. perfringens* type A food poisoning (17) or Darmbrand (this study). Interestingly, the one pigbel isolate (CN5388) examined in a previous investigation did not form highly heat-resistant spores (19), even though pigbel is a food-borne disease considered similar to Darmbrand. However, more pigbel isolates, which are very difficult to obtain, should be examined before any conclusions can be drawn regarding the relative heat resistance characteristics of the spores of pigbel strains.

Carriage of an *ssp4* gene that encodes the Asp36 Ssp4 variant was not the only identified resemblance between Darmbrand strains and type A strains carrying a chromosomal *cpe* gene. While most *C. perfringens* strains carry a chromosomal *pfoA* gene that encodes perfringolysin O (27), both Darmbrand strains and type A strains carrying a chromosomal *cpe* gene are *pfoA* negative (10).

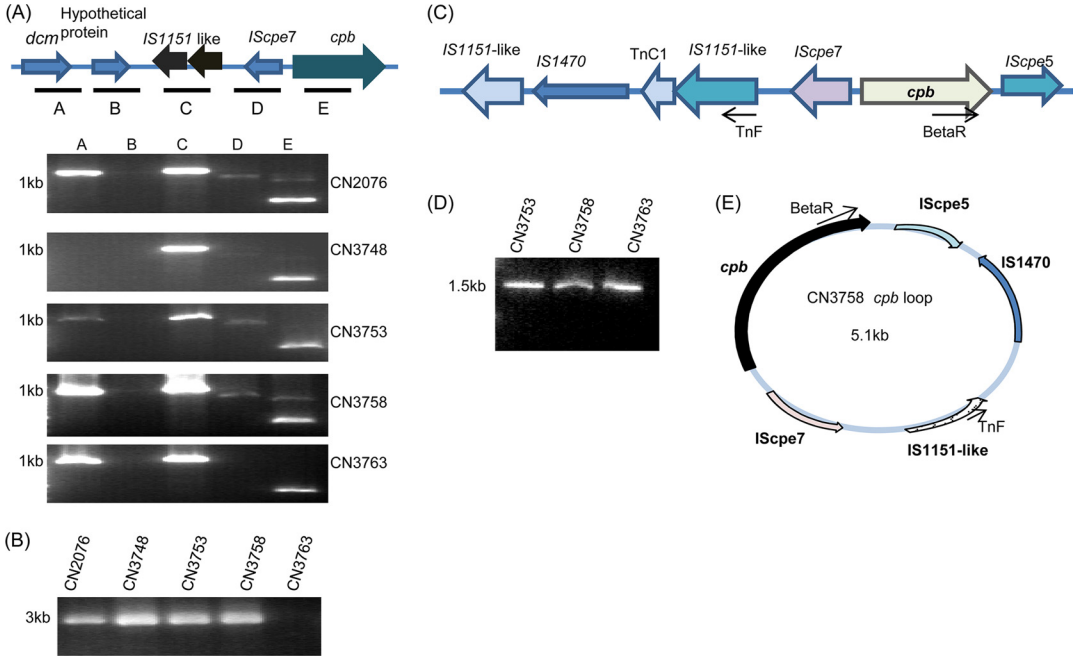

FIG 7 Organization of the *cpb* locus and evidence of *cpb* gene mobilization. (A) Overlapping-PCR linkage of the *cpb* gene with IS*1151* and the *dcm* gene in five type C Darmbrand isolates. The top panel shows a diagram of the *cpb* locus in *C. perfringens* type C isolate JGS1495 based upon the sequencing results released by the J. Craig Venter Institute;. Boxes A to E depict the overlapping PCRs of the panel below. The migration of a 1-kb size marker is shown at the left of the gels. (B) Long-range PCR analysis of the five type C Darmbrand isolates, i.e., CN2076, CN3748, CN3753, CN3758, and CN3763, using primers F3/R5. The size of the PCR product is shown at the left of the gel. (C) Arrangement of the *cpb* locus in strains CN2076, CN3748, CN3753, and CN3758 based upon our sequencing data. The reverse arrows show the orientation and binding of primers used for loop PCR. (D) PCR amplification of *cpb*-containing circular intermediates using primer pair TnF/BetaR with CN3753, CN3758, and CN3763 DNA extracted freshly from colonies grown overnight anaerobically on BHI agar plates. The migration of a 1-kb size marker is shown at the left lane of the gel. (E) Diagram of the circular intermediate derived from sequencing of the CN3758 loop PCR product in panel D that was amplified using primers TnF and BetaR.

Moreover, MLST analyses of eight housekeeping genes revealed that the genetic similarities between Darmbrand strains and type A food poisoning strains carrying a chromosomal *cpe* gene are even more pervasive than the simple absence of *pfoA* or the encoding of an Asp36 Ssp4 variant. By collating results from the present and previous (10) MLST studies, Darmbrand and type A chromosomal *cpe*-positive strains were found to group closely together on the *C. perfringens* phylogenetic tree; moreover, these

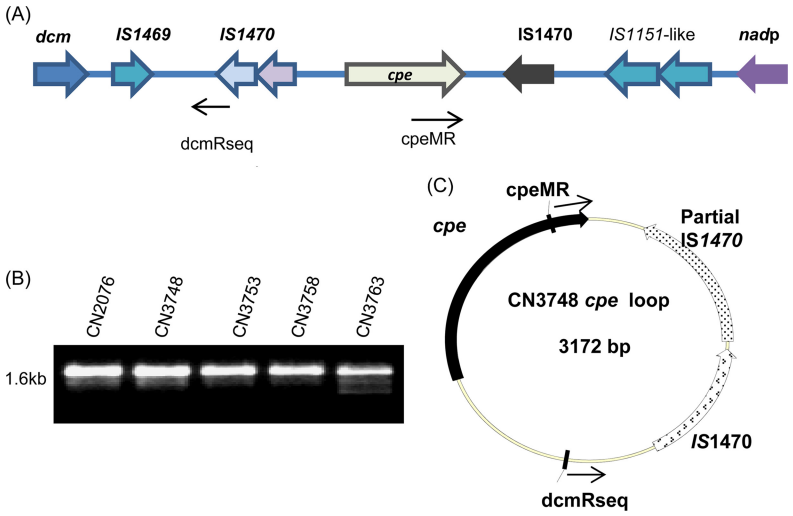

FIG 8 Organization of the *cpe* gene locus and evidence of *cpe* gene mobilization. (A) Diagram of the *cpe* locus organization in isolate CN3748. The reverse arrows show the orientation and binding of primers dcmRseq and cpeMR used for loop PCR in panel B. (B) PCR amplification of *cpe*-containing circular intermediates using primer pair dcmRseq/cpeMR with CN2076, CN3748, CN3753, CN3758, and CN3763 DNA extracted freshly from colonies grown overnight anaerobically on BHI agar plates. The migration of a 1-kb size marker is shown to the left of the gel. (C) Diagram of the circular intermediate derived from sequencing of the CN3748 loop PCR product in panel B that was amplified using primer set dcmRseq/cpeMR.

strains have a genetic background distinctly different from that of plasmid *cpe*-positive type A to E veterinary strains. Therefore, Darmbrand and type A chromosomal *cpe*-positive strains represent a distinct subpopulation within the *C. perfringens* species. It should also be noted that the only other Darmbrand isolate (NCTC8081) that had been previously characterized by MLST (10) is also classified in this phylogenetic subgroup.

Darmbrand is usually attributed to type C strains of *C. perfringens* (13, 14). Therefore, it was notable that two of the seven Darmbrand isolates surveyed in this study were genotyped instead as type A. At least two explanations for these results are conceivable. First, these two type A Darmbrand strains may have been merely normal-flora *C. perfringens* strains that were isolated from Darmbrand patients, rather than the strains that actually caused the illness. This possibility seems less likely since the type A Darmbrand strains were genetically similar to type C Darmbrand strains with respect to their MLST results, the absence of a *pfoA* gene, the carriage of an *ssp4* gene that encodes the Asp36 Ssp4 variant, and the production of highly heat-resistant spores. The alternative explanation is that these Darmbrand strains were originally type C but during the past 60+ years since their collection, they had lost their *cpb* plasmid. Similar "type degradation" has often been reported in the *C. perfringens* literature (23).

It was also notable that all of the type C Darmbrand strains surveyed produced CPE. To our knowledge, CPE production has not been previously associated with Darmbrand, which occurred before the identification of CPE in the late 1960s to the early 1970s (23). However, CPE has been proposed as a possible contributor to the pathogenesis of human pigbel (14), which is also an enteritis necroticans caused by type C strains. The relative importance of CPB versus CPE for the pathogenicity of Darmbrand or other CPE-positive type C strains is not clear at present, although both toxins are clearly active in the gastrointestinal tract and can also induce lethal enterotoxemia (5, 35).

The *cpe* locus present in the five type C Darmbrand strains surveyed matched that reported previously for type C Darmbrand strain CN2078 (18). This plasmid-borne *cpe* locus of Darmbrand strains is also remarkably similar to the *cpe* locus present in type A chromosomal strains (3, 24, 26), except that, in Darmbrand strains, the IS*1469* element is present upstream (rather than downstream) of the IS*1470* element located upstream of the *cpe* gene. These findings, plus the genetic relatedness between Darmbrand strains and type A chromosomal *cpe*-positive strains as revealed by MLST, suggest evolutionary relationships between these strains. Several scenarios could be envisioned for this relationship. First, many toxin plasmids of *C. perfringens* are conjugative (1, 4, 12), so a *C. perfringens* strain with a housekeeping and spore resistance genetic background similar to that now found in Darmbrand and type A chromosomal *cpe*-positive strains may have conjugatively acquired a *cpe*-bearing plasmid like those now found in the Darmbrand strains, creating a Darmbrand precursor strain. Since this study found evidence of mobilization of the *cpe* gene by the adjacent IS sequences in Darmbrand strains, the *cpe* gene in one bacterium of that Darmbrand precursor strain might later have been inserted or recombined into the chromosome to give rise to type A chromosomal *cpe*-positive strains (Fig. 9). Another bacterium of the Darmbrand precursor strain may also have later conjugatively acquired a *cpb* plasmid, which converted it to a type C Darmbrand strain. The present and previous studies (11, 18) identified some Darmbrand strains that carry both their *cpb* and *cpe* genes on similar-sized plasmids, and a previous investigation provided (11) evidence that in at least two such Darmbrand strains (CN3753 and CN2078) those *cpb* and *cpe* genes are located on the same plasmid. Since this study found evidence of the mobilization of both the *cpe* and *cpb* genes in Darmbrand strains, it seems likely that, in some Darmbrand strains, a mobilized toxin gene was later inserted into a plasmid already carrying the other toxin gene; e.g., a mobilized *cpb* gene was inserted into a *cpe*-bearing plasmid or vice versa. An alternative scenario explaining the evolutionary relationship between Darmbrand strains and type A chromosomal *cpe*-positive strains is that the *cpe* gene on the chromosome of a type A strain, which can also be mobilized (3), may have moved onto a plasmid and then that strain later acquired a *cpb*-bearing plasmid by conjugation (Fig. 9).

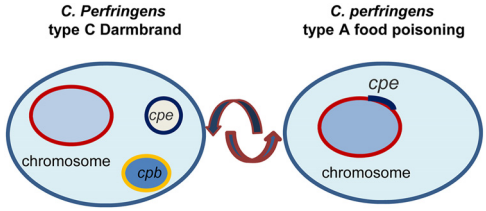

FIG 9 Possible evolutionary relationship between *C. perfringens* type A food poisoning isolates carrying a chromosomal *cpe* gene and type C Darmbrand isolates carrying a plasmid *cpe* gene.

Further research is under way to better understand the relationship between the *cpe*- and *cpb*-bearing plasmids found in various *C. perfringens* strains. This information will shed further light on the diversity and evolutionary relationships between these strains. Studies are also under way to better dissect the relative contributions of CPB and CPE to the pathogenicity of CPE-positive type C strains.

ACKNOWLEDGMENTS

This research was generously supported by grant R01 AI056177-09 from the National Institute of Allergy and Infectious Diseases.

We thank Paul Hauer for supplying a CPB-specific monoclonal antibody.

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