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Virulence Plasmid Diversity in *Clostridium perfringens* Type D Isolates[▽]†

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Clostridium perfringens **type D isolates are important in biodefense and also cause natural enterotoxemias in sheep, goats, and occasionally cattle. In these isolates, the gene (*etx*) encoding ε-toxin is thought to reside on poorly characterized large plasmids. Type D isolates sometimes also produce other potentially plasmid-encoded toxins, including *C. perfringens* enterotoxin and beta2 toxin, encoded by the *cpe* and *cbp2* genes, respectively. In the current study we demonstrated that the *etx*, *cpe*, and *cpb2* genes are carried on plasmids in type D isolates and characterized the toxin-encoding plasmids to obtain insight into their genetic organization, potential transferability, and diversity. Southern blotting of pulsed-field gels showed that the *etx* gene of type D isolates can be present on at least five different plasmids, whose sizes range from 48 to 110 kb. The *etx* plasmids also typically carried IS*1151* and *tcp* open reading frames (ORFs) known to mediate conjugative transfer of *C. perfringens* plasmid pCW3. PCR studies revealed that other than their *tcp* ORFs, *etx* plasmids of type D isolates do not carry substantial portions of the conserved or variable regions in the *cpe* plasmids of type A isolates. Southern blotting also demonstrated that in type D isolates the *cpe* and *cpb2* genes are sometimes present on the *etx* plasmid. Collectively, these findings confirmed that the virulence of type D isolates is heavily plasmid dependent and indicated that (i) a single type D isolate can carry multiple virulence plasmids, (ii) a single type D virulence plasmid can carry up to three different toxin genes, and (iii) many *etx* plasmids should be capable of conjugative transfer.**

The anaerobic sporeformer *Clostridium perfringens* is an important human and animal pathogen (16, 22, 24) that can produce up to 15 different toxins and enzymes. Production of four toxins (α, β, ε, and ι toxins) is used to classify *C. perfringens* into five different types (types A to E) (22). Type D isolates must produce both α-toxin and ε-toxin, which is encoded by the *etx* gene and is the third-most-potent clostridial toxin after the botulinum toxins and tetanus toxin (27). Because of this potency, both the CDC and U.S. Department of Agriculture have listed ε-toxin as an overlap class B select toxin. Beyond their biodefense significance due to their production of ε-toxin, type D isolates also naturally cause enterotoxemias in sheep, goats, and occasionally cattle (16).

Many *C. perfringens* toxins, including ε-toxin, can be plasmid encoded (24). However, despite their biodefense and pathogenic importance, in general, the toxin-encoding *C. perfringens* virulence plasmids have received only limited research attention to date. The major exceptions have been the ~70- to 75-kb plasmids carrying the *cpe* gene (encoding *C. perfringens* enterotoxin) that are present in some type A isolates. Miyamoto et al. recently identified two major families of *cpe*-carrying plasmids in type A isolates, one with an IS*1151* sequence (but no IS*1470*-like sequence) downstream of the *cpe* gene and the other with an IS*1470*-like sequence (but no IS*1151* sequence) downstream of the *cpe* gene (18, 19). These two type A *cpe* plasmid families share a conserved region comprising ~50% of each plasmid. In this conserved region are Tn*916*-related sequences, designated the *tcp* locus (1), that were recently demonstrated to mediate conjugative transfer of the *C. perfringens* tetracycline resistance plasmid pCW3 (1). The presence of this *tcp* locus in the *cpe* plasmids of type A isolates probably explains why some, if not all, of these *cpe* plasmids can transfer conjugatively (5). The variable regions of the two type A *cpe* plasmid families differ significantly and can carry an additional toxin gene (the *cpb2* gene encoding beta2 toxin is present in these *cpe* plasmids with downstream IS*1151* sequences), bacteriocin open reading frames (ORFs), or putative metabolic ORFs (10, 19).

By contrast, much less is known about *etx* genetics. The *etx* gene has been sequenced (12) for only a single type B strain (NCTC8533) and a single type D strain (NCTC8346), and the limited sequencing studies suggested that there may be a few type-specific differences in the *etx* ORF. It has also been shown that insertion sequences (IS) are present upstream and downstream of *etx* in one type D isolate (8, 24; GenBank accession number X60694). Initial studies of a few type D isolates suggested that the *etx* gene is present in a large plasmid (2, 9, 23), but the *etx* plasmid(s) has not been studied yet in any detail. Finally, we and other workers recently showed by using multiplex PCR that there are genotypic variations among type D isolates (26) and that some type D isolates carry up to three different potentially plasmid-borne toxin genes (*etx*, *cpe*, and *cpb2*).

Considering the limited information now available about *etx* genetics, in the current study we sought to confirm whether *etx* genes are typically plasmid borne in a large collection of type D animal disease isolates. If *etx* was confirmed to be predominantly plasmid borne, we then intended to evaluate the diversity of the type D *etx* plasmids and also determine the association, if any, between *cpe*, *cpb2*, and *etx* genes on type D virulence plasmids. Finally, to begin assessing possible mobilization of the *etx* gene or *etx*-carrying plasmids, we examined

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TABLE 1. Plasmid carriage of *etx*, *tcpF*/*H*, and IS*1151* in *cpe*-negative *cpb2*-negative type D isolates

Isolate	Size (kb) of plasmid carrying:		
	etx	*tcpF*/*H*	IS*1151*
CN462	48	48	48
CN1020	48	48	48
CN1184	48	48	48
CN1634	75	48	75
CN2062	48	48	48
CN3693	48	48	48
CN3793	48	48	48
CN3977	48	48	48
CN3978	48	48	48
CN3841	48	48	48
CN4029	75	75	75
CN4031	75	75	75
JGS1942	48	48	48

TABLE 2. Plasmid carriage of selected genes in *cpe*$^+$ and/or *cpb2*$^+$ type D isolates

Isolate	Size(s) (kb) of plasmid(s) carrying:				
	etx	*cpe*	*cpb2*	*tcpF*/*H*	IS*1151*
CN2068	73		73	73	73
CN1183	75	75	75	75	75
CN3842	85	85	85	85	85
CN4003	75	110	45	75, 110	110
CN3948	75	110	75	75, 110	75, 110
JGS1240	75	110	75	48, 65, 75	48, 65, 75
JGS1902	110	110	75	75, 110	75
JGS4138	110	110	75	75, 110	75, 110
JGS4139	75	110	75	75	75, 110
JGS4152	110	110	75	110	110

whether *tcp* genes or IS sequences are typically present on *etx* plasmids.

MATERIALS AND METHODS

Bacterial strains, media, and reagents. As described previously (26), the 23 *C. perfringens* type D isolates examined in this study originated from diseased animals. The plasmid-borne toxin genotypes of these isolates (17), also demonstrated previously (26), are shown in Tables 1 and 2. All type D isolates were grown overnight at 37°C under anaerobic conditions on SFP agar (Difco Laboratories) containing 0.04% D-cycloserine (Sigma Aldrich) in order to ensure culture purity. Fluid thioglycolate medium (FTG) (Difco Laboratories) and 3% tryptic soy broth (Becton-Dickinson) containing 2% glucose (Sigma Aldrich), 1% yeast extract (Becton Dickinson), and 0.1% thioglycolate (Sigma Aldrich) (TGY) were used to grow broth cultures.

Pulsed-field gel electrophoresis. *C. perfringens* type D isolates were grown overnight at 37°C in FTG broth. A 0.1-ml aliquot of each of the starter cultures was then inoculated into 10 ml of TGY and grown overnight at 37°C. The overnight TGY cultures were used to prepare genomic *C. perfringens* DNA-containing agarose plugs. The bacterial cells were washed three times in TES buffer (1 M Tris, 0.5 M EDTA, 6.7% [vol/vol] 5 M sucrose; pH 8.0). Cells were resuspended in 0.4 ml of Tris-EDTA and embedded in 2% chromosome-grade agarose (Bio-Rad Laboratories) by mixing equal volumes (0.4 ml) of a cell suspension and melted agarose equilibrated to 65 to 70°C. Plugs were solidified at 4°C in 1.5-mm-thick molds (Bio-Rad Laboratories) and then cut into 2- to 3-mm slices. The agarose-embedded cells were lysed by incubation with gentle shaking of the plugs overnight at 37°C in lysis buffer (0.5 M EDTA [pH 8.0], 2.5% [vol/vol] 20% Sarkosyl, 0.25% lysozyme [Sigma], 0.2% deoxycholic acid). Finally, the plugs were incubated in 0.2% proteinase K (Gene Choice) buffer at 55°C for 2 days.

To help distinguish whether colocalizing signals obtained using probes for two different ORFs indicated that these two ORFs were present on the same plasmid or on two comigrating plasmids, restriction endonucleases ApaI, BamHI, BstXI, ClaI, KpnI, NcoI, SalI, ScaI, SmaI, StuI, and XhoI (New England Biolabs) were used to digest plugs containing DNA from two representative type D isolates (CN1183 and JGS1902). In these experiments, each set of plugs was incubated with or without a restriction enzyme in 200 μl of the appropriate buffer solution, as recommended by the enzyme manufacturer.

Pulsed-field gel electrophoresis was performed with a 1% agarose gel using a CHEF-DR II system (Bio-Rad Laboratories) and 0.5× Tris-borate-EDTA buffer at 14°C. The running parameters for undigested DNA were as follows: initial pulse, 1 s; final pulse, 25 s; voltage, 6 V/cm; time, 24 h. The following running parameters were used for DNA digested with restriction enzymes: initial pulse, 1 s; final pulse, 12 s; voltage, 6 V/cm; time, 15 h. After pulsed-field gel electrophoresis, the gel was stained with ethidium bromide, washed with distilled water, and photographed under UV light. Mid-Range or Low-Range PFG markers (New England Biolabs) were used as molecular size standards, as appropriate.

Southern hybridization. Digoxigenin (DIG)-labeled probes were prepared using the primers listed in Table SI in the supplemental material. Southern hybridization of pulsed-field gels was performed as described previously (19). DIG labeling and detection reagents were obtained from Roche Applied Science. The CSPD substrate (Roche Applied Science) was used for detection of hybridized probes according to the manufacturer's instructions.

PCR analyses. Template DNA for all PCRs was obtained from colony lysates, which were prepared as described previously (28). Each PCR mixture contained 5 μl of template DNA, 40 μl of TAQ Complete 1.1 Master Mix (Gene Choice), and 2.5 μl of each primer pair (final concentration, 1 μM). The primers used to investigate whether the variable region or the *tcp* locus of the conserved region in type A *cpe* plasmids pCPF4969 and pCPF5603 are also present in type D isolates were described previously by Miyamoto et al. (19). New overlapping PCR primers were designed for the remaining conserved region (~20 kb) of pCPF5603 (see Table SII in the supplemental material), and these primers were used to investigate whether the entire conserved region of pCPF5603 is present in type D isolates. PCRs were performed with a Techne (Burkhardtsdorf, Germany) thermocycler using previously described conditions (19). PCR products were separated on 1% agarose gels and visualized with ethidium bromide staining.

The presence of the *lam* gene encoding lambda toxin or the *rep* gene encoding the Rep protein of pCW3 (1) was assessed using a primer set shown in Table SI in the supplemental material. The PCR conditions used for amplification of the *lam* gene were as follows: 94°C for 3 min and then 35 cycles of 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min. The PCR products were electrophoresed on a 1% gel and stained with ethidium bromide for visualization.

PCR analysis of the association between *etx* and ISs. Template DNAs used for the PCR analysis of the association between *etx* and ISs were prepared as described above. Internal amplification of IS*1151* was performed using the following primer set, which should have yielded a 638-bp PCR product: IS1151F (5′-CTGTACGGCTCCATTATCTC-3′) and reverse primer IS1151R (5′-CAG TAAGTTCAATTGTTTCGCC-3′). Attempts were also made to connect IS*1151* to the *etx* gene using the following primer set: IS1151F1 (5′-GTTAAATTAGA GCGATTCATGTGC-3′) and etxR2 (5′-CCACTTACTTGTCCTACTAAC-3′). The following PCR primers were used to amplify the downstream (IS*406*) transposase detected previously near the *etx* gene of one type D isolate (GenBank accession number X60694): etx-dnR (5′-CTTCATCAGTAGGAAAAGCTG-3′) and etx-dnF (5′-GGAAATGTAAAGTTAGTAGGAC-3′). The PCR conditions used for amplification were as follows: 94°C for 3 min and then 35 cycles of 94°C for 1 min, 55°C for 1 min, and 72°C for 2 min. The PCR products were electrophoresed on a 1% gel and stained with ethidium bromide for visualization.

Sequencing of the *etx* gene. DNA for sequencing was prepared as described previously (18). The following three forward primers were used for sequencing the *etx* ORF in selected type D isolates: extF1 (5′-GTTTTAAAATACAAGTT TTATG-3′), extF2 (5′-GAGAAAGGAAGATATAATAC-3′), and extF3 (5′-C TAGTTATAGTTTTGCAAATAC-3′). The two reverse primers used for sequencing were etxR1 (5′-GACCTAACTTTACATTTC-3′) and etxR2 (5′-CCA CTTACTTGTCCTACTAAC-3′). Sequencing was performed at the University of Pittsburgh core sequencing facility (http://www.genetics.pitt.edu/services .html), and the sequences were then analyzed using BioEdit and BLAST (NCIB).

PCR identification of possible circular transposition intermediates. Template DNAs used for PCR identification of possible circular transposition intermediates were prepared as described previously (28). Amplification of possible circular transposition intermediates containing only IS*1151* and the *etx* ORF was performed using the following primer set, which should have yielded an ~1.1-kb PCR product: 1R (5′-CTGTTATACTGCCTTTTCTTTG-3′) and 3F (5′-CAC AAGATATACTAGTACCAGC-3′). Amplification of a second possible circular transposition intermediate containing IS*406*, the *etx* ORF, and IS*1151* was per-

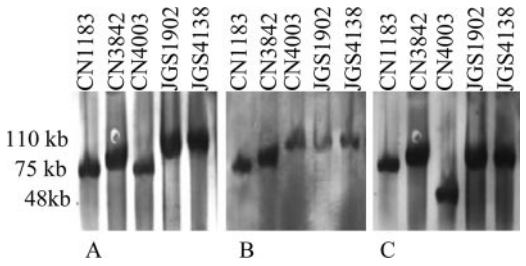

FIG. 1. Southern blot comparison of pulsed-field gels electrophoresed with DNA from *cpe*$^+$ and/or *cpb2*$^+$ type D isolates. The blot was first hybridized with a DIG-labeled, *etx*-specific probe (A) and then stripped and reprobed with a DIG-labeled, *cpe*-specific probe (B). Finally, the blot was striped again and reprobed with a DIG-labeled, *cpb2*-specific probe (C). The positions of molecular size markers are indicated on the left, and isolate designations are indicated above the lanes.

formed using the following primer set, which should have yielded a ~1.6-kb PCR product: 4F (5′-GAAAGGCATGTCTACACGAG-3′) and 2R (5′-CCATGGC CGTCAACCTAAG-3′). Finally, amplification of a third possible circular transposition intermediate containing only IS*406* and the *etx* ORF was performed using the following primer set, which should have yielded a ~127-bp PCR product: 4F (5′-GAAAGGCATGTCTACACGAG-3′) and 1R (5′-CTGTTATA CTGCCTTTTCTTTG-3′). The PCR conditions used for these amplifications were as follows: 94°C for 3 min and then 35 cycles of 94°C for 1 min, 55°C for 1 min, and 72°C for 2 min. The PCR products were electrophoresed on a 1% gel and stained with ethidium bromide for visualization. The PCR products were cloned into the pCR2.1-TOPO vector (Invitrogen, Carlsbad, CA) and sequenced using vector-specific primers M13F and M13R.

Western immunoblotting. Type D isolates were first grown in FTG, and then a 0.1-ml aliquot of each overnight culture was transferred into 10 ml of TGY and grown overnight. Samples were collected, and supernatants were then mixed with an equal volume of loading buffer and electrophoresed in a 10% polyacrylamide gel. The separated protein bands were transferred to a nitrocellulose membrane, and Western blotting for detection of ε-toxin was performed as described previously (26).

RESULTS

Characterization of type D *etx* plasmid diversity and toxin gene carriage. Previous studies have shown that *C. perfringens* chromosomal genes do not enter pulsed-field gels unless the genes are digested with restriction endonucleases (7, 9, 10, 14). However, their smaller sizes allow even undigested plasmids to enter pulsed-field gels. Furthermore, studies with other *C. perfringens* plasmids have shown that on Southern blots of pulsed-field gels, probes react mainly with the nicked, linear forms of plasmids (7, 9, 10, 14, 19). Therefore, their migration on pulsed-field Southern blots provides an accurate estimate of the size of *C. perfringens* plasmids. This conclusion is supported by the results shown in Fig. SIB in the supplemental material for pCPF5603, which indicated that the *cpe* plasmid was ~75 kb in size, which matched the sequencing-determined size of this type A *cpe* plasmid (19).

When this pulsed-field gel Southern blot approach was used to evaluate the location of the *etx* gene (chromosomal versus plasmid) in a collection of type D isolates, three isolates (e.g., CN1675 in Fig. SIB in the supplemental material) showed smearing, which indicated that their nuclease levels were high (7), and they could not be analyzed by this technique. All of the remaining 23 type D isolates surveyed were found to carry a plasmid-borne *etx* gene; i.e., their DNA hybridized with the *etx* probe entered pulsed-field gels without any restriction endonuclease digestion. For type D isolates with a simple *plc* and *etx* toxin genotype (i.e., neither the *cpe* gene nor the *cpb2* gene was present), the *etx* gene was generally present on an ~48-kb plasmid, although a few such type D isolates carried the *etx* gene on larger plasmids (~73 to 75 kb) (see Fig. SIA in the supplemental; Table 1). Type D isolates with a more complicated toxin genotype (*cpe*$^+$ and/or *cpb2*$^+$) carried the *etx* gene on large plasmids that ranged from ~75 to 110 kb in size (Fig. 1 and Table 2; see Fig. SIB in the supplemental material). In these type D isolates, the *cpe* or *cpb2* toxin genes were also present on plasmids, which varied from ~48 to 110 kb in size. For some *cpe*- and/or *cpb2*-positive type D isolates (e.g., CN1183 and CN3842), the *etx*, *cpe*, and *cpb2* probes all hybridized to the same Southern blot location (Fig. 1 and Table 2). However, for other *cpe*- and/or *cpb2*-positive type D isolates, the *etx* probe and the *cpe* or *cpb2* probes hybridized to different plasmids. For example, type D isolate CN4003 clearly carries three distinct toxin-encoding plasmids, including an ~75-kb *etx* plasmid, an ~110-kb *cpe* plasmid, and an ~48-kb *cpb2* plasmid (Fig. 1).

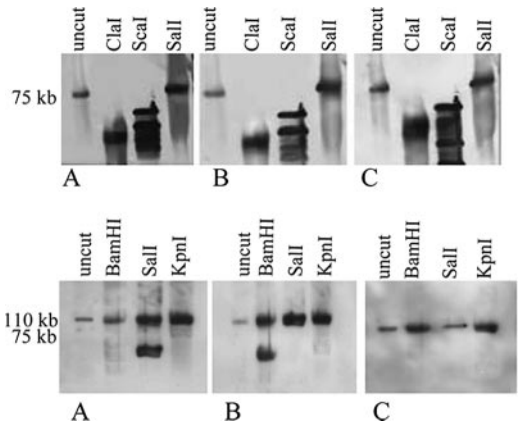

FIG. 2. Southern blot analysis of pulsed-field gels electrophoresed with DNA from CN1183 (top panel) or JGS1902 (bottom panel) that had been digested with specified restriction enzymes. Each Southern blot was first hybridized with a DIG-labeled, *etx*-specific probe (A) and then stripped and reprobed with a DIG-labeled, *cpe*-specific probe (B) before it was stripped and probed with a DIG-labeled, *cpb2*-specific probe (C). The positions of molecular size markers are indicated on the left, and the restriction enzymes used are indicated above the lanes.

Evaluation of whether comigrating toxin genes are present on the same or different plasmids. As shown in Fig. 1, DNA from several type D isolates hybridized to multiple toxin gene probes at the same blot location, indicating that these isolates carry multiple toxin genes on either the same plasmid or on two distinct plasmids whose sizes are similar. To discriminate between these two possibilities, four representative type D isolates were digested with a battery of restriction endonucleases. For type D isolates CN1183 (Fig. 2) and CN3842 (not shown), migration of the *etx* gene, migration of the *cpe* gene, and migration of the *cpb2* gene all exhibited similar susceptibility to restriction enzyme digestion, a result consistent with the hypothesis that these three toxin genes are located on the same plasmid (Fig. 2 and Table 2). However, for type D isolate JGS1902 (Fig. 2), there were differences in the digestion susceptibility patterns for the plasmid DNA carrying the *etx* gene

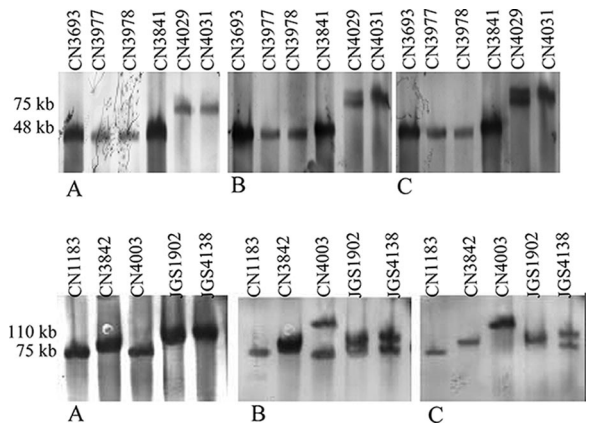

FIG. 3. Southern blot analysis of pulsed-field gels electrophoresed with DNA from *cpe*-negative *cpb2*-negative (top panel) or *cpe*$^+$ and/or *cpb2*$^+$ (bottom panel) type D isolates and hybridized with a DIG-labeled, *etx*-specific probe (A). The blot was then stripped and reprobed with a DIG-labeled, *tcpF*- and *tcpH*-specific probe (B) before it was stripped and probed with a DIG-labeled IS*1151*-specific probe (C). The positions of molecular size markers are indicated on the left, and isolate designations are indicated above the lanes.

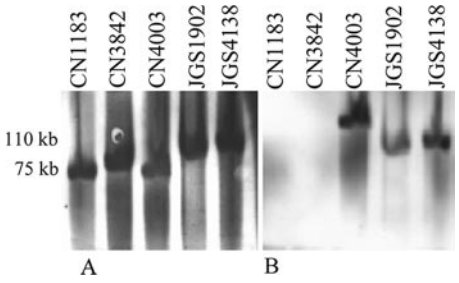

FIG. 4. Southern blot analysis of pulsed-field gels electrophoresed with DNA from CN1183, CN3842, CN4003, JGS1902, and JGS4138. The Southern blot was first hybridized with a DIG-labeled, *etx*-specific probe (A) and then stripped and reprobed with a DIG-labeled, *lam*-specific probe (B). The positions of molecular size markers are indicated on the left.

and the plasmid DNA carrying the *cpe* gene, suggesting that the *etx* and *cpe* genes in this isolate may be on two distinct ~110-kb plasmids. In this isolate the *cpb2* gene is present on a smaller (75-kb) plasmid (Fig. 1). For type D isolate JGS4138 (data not shown), there was no difference between the digestion susceptibility pattern for the plasmid DNA carrying the *etx* gene and the digestion susceptibility pattern for the plasmid DNA carrying the *cpe* gene, suggesting that the *etx* and *cpe* genes of this strain are present on the same ~110-kb plasmid. In JGS4138 the *cpb2* gene is present on a smaller (75-kb) plasmid (Fig. 1).

Presence of the *tcp* transfer locus in type D isolates. In previous studies, (1, 19) workers reported that *tcp* genes, which mediate conjugative transfer of *C. perfringens* plasmid pCW3 (and probably many *cpe* plasmids of type A isolates [5, 19]), are present in type D isolate NCTC8346 and a second, unspecified type D isolate. To investigate whether *tcp* genes are commonly found in type D isolates and, if so, whether they are present on *etx* plasmids or other virulence plasmids in these isolates, Southern blot analyses of pulsed-field gels were performed using probes specific for two *tcp* genes (*tcpF* and *tcpH*) known to be required for pCW3 conjugative plasmid transfer (1). In most type D isolates surveyed, *tcpF* and *tcpH* probes hybridized to the same blot location containing the *etx* plasmid (Fig. 3 and Tables 1 and 2). However, in isolate CN1634, *tcpF* and *tcpH* probes did not hybridize with the *etx* plasmid, although they did appear to hybridize to a smaller ~48-kb non-toxin-encoding plasmid (data not shown). These *tcp* Southern blot analyses also strongly suggested that type D isolates JGS1902 and JGS4138 carry a distinct *cpb2* plasmid with *tcpF* and *tcpH* genes (Fig. 3).

PCR analysis also detected the presence of the gene encoding the pCW3 Rep protein (1), which is also present in pCPF4969 and pCPF5603 (19), in all 10 type D isolates surveyed. Southern blots of pulsed-field gels containing DNA from seven of these type D isolates showed that there was comigration of the hybridized *rep* and *etx* probes, suggesting that *rep* is commonly present on *etx* plasmids (data not shown).

Presence of the lambda toxin gene in type D isolates. Lambda toxin is a *C. perfringens* metalloprotease that can proteolytically activate ε-toxin (13, 20). Since the lambda toxin gene (*lam*) has been detected previously (13) in two type D isolates (945P and NCTC2062), we PCR tested our 23 type D isolates and 15 other type D isolates to determine whether they carried the *lam* gene. This survey detected the *lam* gene in 9 of the 38 type D isolates. To confirm these results and to assess whether the *lam* gene is plasmid borne in *lam*$^+$ type D isolates, we performed a Southern blot analysis of pulsed-field gels. In this analysis (Fig. 4) we detected no *lam* probe hybridization with DNA from two type D isolates that were PCR negative for the *lam* gene. In contrast, DNA from three *lam* PCR-positive type D isolates hybridized with the *lam* probe. For one of these three type D isolates, the *lam* gene was clearly not present on the *etx* plasmid but instead appeared to be present on a larger, non-toxin-encoding plasmid. For the other two type D isolates, the *lam* probe apparently hybridized with the *etx* plasmid.

We also used Western blotting to compare ε-toxin processing by *lam*$^+$ and *lam*-negative type D isolates. Proteolytic processing of ε-toxin was sometimes evident (data not shown) in supernatants from one of eight *lam*$^+$ isolates. Interestingly, two of four *lam*-negative isolates also showed intermittent ε-toxin processing (data not shown).

PCR studies to evaluate whether the variable or conserved regions of pCPF5603 or pCPF4969 are present in type D isolates. As mentioned above, two major *cpe* plasmid families have been identified in type A isolates, and a representative of each family (pCPF4969 and pCPF5603) has now been completely sequenced (19). Since *tcp* genes are present in pCPF5603, pCPF4969, and most type D isolates (19) (Fig. 3), PCR analyses were performed to evaluate the possible presence of other pCPF4969 or pCPF5603 genes in type D isolates. Individual ORF PCR surveys (see Table SIII in the supplemental material) revealed that most type D isolates carry many ORFs found in the conserved region of pCPF4969 and pCPF5603. However, with the exception of type D isolates CN4003 and JGS1182, individual ORFs of either the pCPF5603 or pCPF4969 variable region were not found in the type D isolates surveyed.

Overlapping PCR primer sets were then employed to eval-

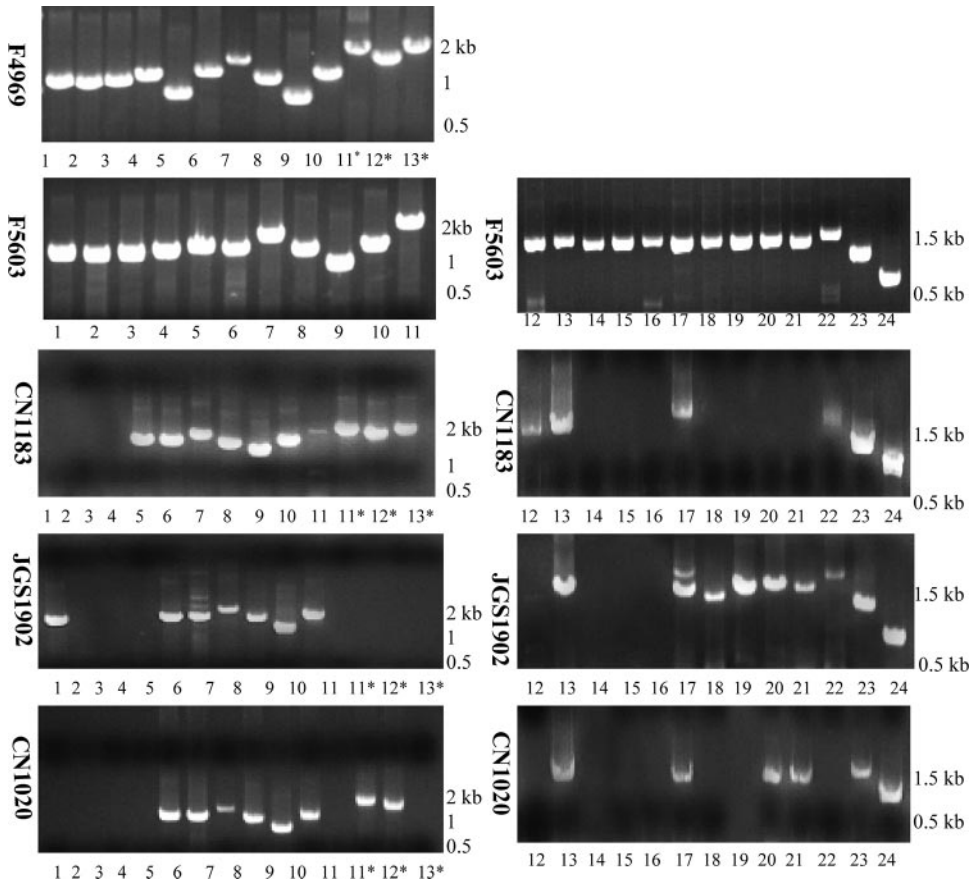

FIG. 5. Conserved region of control *cpe* plasmids pCPF4969 and pCPF5603 from type A isolates PCR amplified with an overlapping PCR battery (19). The same PCR primers and conditions were then used to assess the presence of this region in different type D isolates. The positions of molecular size markers are indicated on the right. Positive reactions 5 to 10 correspond to the *tcp* locus. Isolate designations are indicated on the left, and reaction numbers are indicated below the lanes. Asterisks indicate reactions specific for pCPF4969 sequences (19).

uate whether the conserved region ORFs detected by individual ORF PCRs are arranged similarly in type D isolates and in pCPF4969 and pCPF5603. The *tcp* locus was the only product amplified from the type D isolates surveyed by these overlapping PCR assays (Fig. 5), suggesting that compared to the conserved region of pCPF5603 and pCPF4969, there are substantial ORF rearrangements or sequence differences upstream and downstream of the putative *tcp* transfer region in the *etx* plasmids of type D isolates.

As expected from the individual ORF PCR survey results, overlapping PCR analyses also did not detect (data not shown) the pCPF5603 or pCPF4969 variable region in most type D isolates (although the surveys were positive for type A control plasmids pCPF5603 and pCPF4969 [data not shown]). Even the two type D isolates (JGS1182 and CN4003) that amplified most individual pCPF5603 variable ORFs did not generate positive reactions using the overlap PCR battery for the pCPF5603 variable region (data not shown), indicating that there are substantial ORF rearrangements or sequence differences between the variable region of pCPF5603 and the plasmids in type D isolates JGS1182 or CN4003.

Presence of the IS*1151* sequence in type D isolates. In previous studies, workers have demonstrated that an IS*1151* sequence is located upstream of the *etx* gene in at least one type D isolate (8, 24). Therefore, we surveyed whether there is a similar *etx* locus arrangement in other type D isolates using an overlap PCR capable of linking the *etx* gene and IS*1151* in this previously analyzed type D isolate. This PCR analysis successfully linked an upstream IS*1151* to the *etx* gene in all *cpb2*-negative *cpe*-negative type D isolates surveyed (Fig. 6B). However, the same primer set did not amplify a product from type D isolates also carrying the *cpb2* and/or *cpe* genes, even though these isolates were PCR positive when internal IS*1151* primers were used (Fig. 6A). These results are consistent with pulsed-field gel Southern blot results showing that IS*1151* is variably associated with the *etx* plasmid in the *cpb2*- and/or *cpe*-positive type D isolates (Fig. 3).

In another previous study workers showed that IS*406* sequences (GenBank accession number X60694) were present downstream of the *etx* gene in one type D isolate. In the current study, PCR analysis linked the downstream transposase to the *etx* gene in all type D isolates surveyed irrespective of the toxin genes that they carried (Fig. 6C).

PCR identification of possible circular transposition intermediates. Results shown in Fig. 6 confirmed the previous finding (GenBank accession number X60694) that in *cpe*-negative *cpb2*-negative type D isolates there are two ISs flanking the *etx* gene, suggesting that this locus may represent an integrated

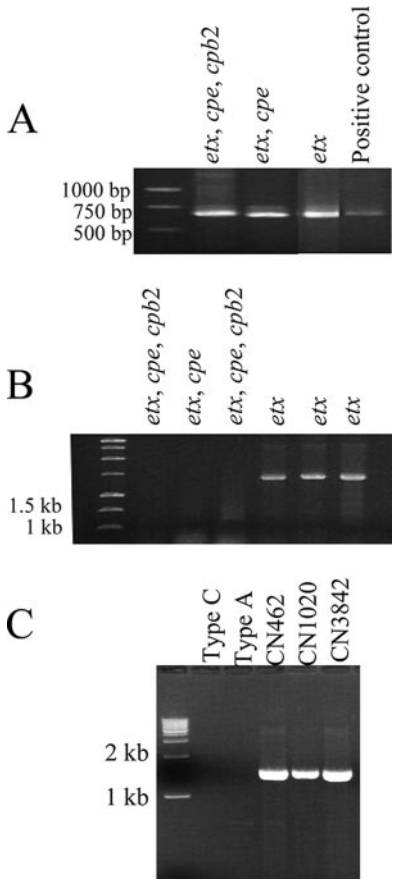

FIG. 6. Presence of insertion sequences in type D isolates. (A) PCR amplification of internal IS*1151* sequences from three different type D isolates. The toxin-borne genes present in each isolate are indicated above the lanes. (B) PCR amplification linking the upstream IS*1151* sequence and the *etx* gene in six different type D isolates. (C) PCR amplification linking the downstream IS*406* sequence and the *etx* gene in three different type D isolates. The positions of molecular size markers are indicated on the left. Genotypes and isolate designations are indicated above the lanes.

genetic element. Movement of ISs and their associated genes often involves formation of small circular transposition intermediates (4). To test whether the ISs near the *etx* gene in *cpe*-negative *cpb2*-negative type D isolates might mobilize this toxin gene into circular intermediates, primers were designed to identify three possible circular transposition intermediates in these isolates (Fig. 7). In these PCR analyses we clearly identified a transposition intermediate including both ISs and the *etx* gene (~3.3 kb). This finding was confirmed by sequencing the PCR product, which contained IS*1151*-*etx*-IS*406* sequences. Primers for other possible circular intermediates also amplified products, but amplification of multiple products in these PCRs precluded sequencing attempts to confirm whether these products represent the expected circular transposition intermediates.

Sequencing of the *etx* ORF in type D isolates. In a previous study (11), workers sequenced the *etx* ORF from one type D isolate (NCTC8346) and one type B isolate (NCTC8533), and the results suggested that there might be type-specific *etx* ORF differences. Therefore, we sequenced the *etx* ORF from eight selected type D isolates, including a mixture of *cpe*-negative *cpb2*-negative type D isolates and type D isolates carrying *cpe* or both *cpe* and *cpb2*. These analyses showed that the *etx* genes in the type D isolates surveyed encode mature ε-toxins with the same amino acid sequence. However, some type D isolates carried the *etx* ORF previously associated with the *etx* ORF of type B isolates; i.e., their *etx* ORF encoded a threonine (rather than serine) at prototoxin amino acid position 321.

DISCUSSION

Type D isolates producing ε-toxin cause natural enterotoxemias of lambs, goats, and other animals when ε-toxin, an overlap CDC/U.S. Department of Agriculture class B select toxin, is absorbed through the intestinal mucosa. The absorbed toxin then spreads via the circulation to different internal organs, such as the kidney and brain, and then causes elevated blood pressure, edema, and neurological signs (16, 21, 25). The mechanism of action of ε-toxin has been studied intensively in the past several years, but very little is known about *etx* genetics.

Our current findings confirm previous suggestions, based on analysis of only a few type D isolates, that the *etx* gene commonly resides on large plasmids (2, 14). However, we discovered considerable variation in *etx* plasmid size. In *cpe*-negative *cpb2*-negative type D isolates, the *etx* plasmid is typically ~48 kb long, but it can be as large as ~73 to 75 kb. In type D isolates that also carry a *cpe* and/or *cpb2* gene, the *etx* plasmid is typically very large, up to ~110 kb in size. In these type D isolates with a more complicated toxin genotype, the *etx*, *cpe*, and *cpb2* genes are sometimes present on the same plasmid; i.e., a single type D plasmid can encode three different lethal toxins. However, *cpb2*-positive type D isolates generally carry the *cpb2* gene on a smaller plasmid distinct from the *etx* plasmid. In fact, results of the current study revealed that it is possible for a simple type D isolate to carry three different toxin plasmids.

The current plasmid analyses also demonstrated that *tcp* locus genes (1) essential for conjugative transfer of pCW3 are also apparently associated with the *etx* plasmid in many type D isolates. Furthermore, our results indicate these *tcp* genes can sometimes also be associated with non-*etx*-containing *cpe* or *cpb2* plasmids in type D isolates; the latter discovery is interesting since in previous studies workers did not identify any *tcp*-carrying *cpe*-negative *cpb2*$^+$ plasmids in type A isolates (19). Since the *tcp* locus can mediate conjugative transfer of pCW3 (1) and probably also mediates conjugative transfer of pCPF4969 and other *cpe* plasmids (5, 19), these findings suggest that most *etx* plasmids should be transferable by conjugation. However, studies are needed to confirm this hypothesis.

Recently, two *cpe*-carrying type A plasmids have been completely sequenced, and the sequence information was used to identify two major type A *cpe* plasmid families that share a conserved region (about 50% of each plasmid) but also carry quite different variable regions (19). When we evaluated (by overlapping and individual PCRs) whether the conserved or variable regions of type A *cpe* plasmids are also present in type D isolates, individual conserved region ORFs of type A *cpe* plasmids were identified in type D isolates. However, the arrangement or sequence of these conserved region ORFs in

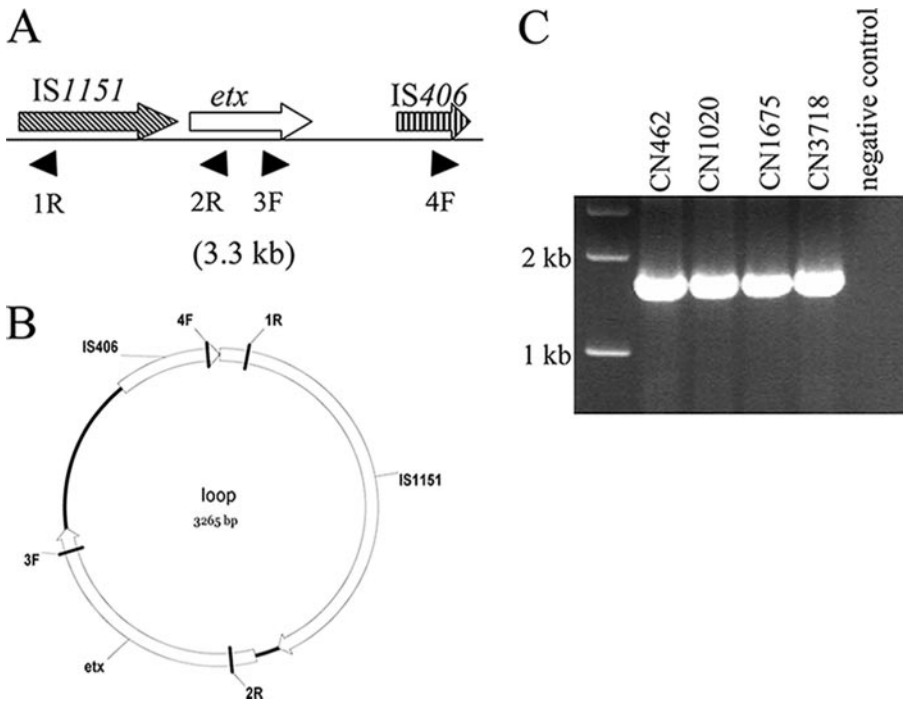

FIG. 7. (A) Arrangement of the *etx* locus in *cpe*-negative *cpb2*-negative type D isolates based on the results shown in Fig. 6. The designations below the line indicate the primers used to evaluate circular intermediate formation. (B) Map of the circular intermediate based on PCR and sequencing (not shown). (C) PCR amplification of the circular intermediate. The positions of molecular size markers are indicated on the left, and isolate designations are indicated above the lanes.

type D isolates must be substantially different from the arrangement or sequence in type A *cpe* plasmids. This suggests that the type A *cpe* plasmids and the type D *etx* plasmids, even when they carry the *cpe* gene, may not be closely related beyond their *tcp* sequences. Further studies are needed to evaluate whether the type A *cpe* plasmid conserved region ORFs detected are present on the *etx* plasmid in type D isolates and, if so, how they are arranged on this plasmid. While two type D isolates did carry substantial type A *cpe* plasmid variable region ORFs, even the two shared ORFs appeared to have substantially different sequences or were arranged differently in these two type D isolates and the type A *cpe* plasmids. Collectively, these findings support the conclusion that sequencing of one or more *etx* plasmids from type D isolates is necessary in order to understand the contributions to virulence and the evolution of these plasmids.

There is emerging evidence that there is a close association between IS sequences and many *C. perfringens* toxin genes (6, 18, 24). For example, IS*1469*, IS*1151*, and IS*1470* sequences are located near the *cpe* genes of type A isolates (4, 6, 18), while IS*1151* sequences are located near the ι-toxin genes in type E isolates (3, 15). It has been suggested that, at least for the chromosomal *cpe* gene (6) and ι-toxin gene (15), these IS sequences can mobilize the adjacent toxin gene, thereby affecting the evolution of virulence of individual *C. perfringens* isolates and, by extension, the entire species. In a previous study the workers identified an IS*1151* sequence upstream of *etx* in a few type D isolates (8, 24), but our findings suggest that there may be a more complicated relationship between IS*1151* and *etx*. While most or all type D isolates carry IS*1151*, this IS was found only immediately upstream of *etx* in *cpe*-negative *cpb2*-negative type D isolates. These findings are the first report of *etx* locus differences among type D isolates. At least in vitro, these *etx* locus variations do not affect ε-toxin expression levels (26; this study), but it is notable that isolates carrying *etx* on a ~110-kb plasmid consistently produced low levels of ε-toxin (26; this study). The current study also showed that in contrast to the variations present upstream of the *etx* gene, most or all type D isolates carry a similar IS*406*-like sequence downstream of the *etx* gene.

Our Southern blot and overlapping PCR results clearly indicate that the *etx* gene occurs on several different plasmids. The association between IS sequences and the *etx* gene in type D strains (9; this study) suggests a possible mechanism to explain this observed *etx* plasmid diversity; i.e., nearby IS sequences could mobilize *etx* sequences from one *etx* plasmid, followed by subsequent insertion of the *etx*-carrying mobile element into a second plasmid. Consistent with this hypothesis, a circular form with a circular transposition intermediate containing IS*406*-*etx*-IS*1151* was identified by PCR in this study. The suggested mobility of the *etx* gene and other toxin genes (9) has implications for the evolution of the virulence of *C. perfringens*; e.g., this could help explain why some type D isolates carry a single plasmid with three different toxin genes.

In our *etx* sequencing study we found that most or all type D isolates have an *etx* gene sequence that is highly conserved at the nucleotide level, as well as the amino acid level. Previously, it was shown that the *etx* gene of one type B isolate encodes an ε-toxin prototoxin with serine at amino acid residue 321, while the *etx* gene of one type D isolate encodes an ε-toxin with a

threonine at this position (11). Our sequencing results indicated that both *etx* ORF variants can be found in type D isolates; i.e., these *etx* ORF variations are not type specific. It should be noted that this single amino acid change does not affect the activity of mature ε-toxin, since it occurs in a portion of the ε-toxin prototoxin that is removed by trypsin or chymotrypsin during the maturation process (20).

ε-Toxin requires proteolytic activation. Lambda toxin is capable of activating ε-toxin (13), but the importance of this effect is unclear since trypsin or chymotrypsin also activates ε-toxin (20). Our results strongly suggest that many type D isolates do not carry the *lam* gene. Since the type D isolates surveyed were associated with animal diseases, this suggests that lambda toxin production may not be required for virulence. This contention is consistent with our recent mouse lethality results (26) indicating that the ε-toxin activity of culture supernatants from most type D isolates, including several isolates surveyed by Western blotting for ε-toxin processing in the current study, is low or nonexistent unless it is activated with trypsin. Even supernatants from *lam*-positive type D isolates contained little or no ε-toxin activity prior to trypsin treatment (26; this study), suggesting either that the *lam* gene of these isolates is not expressed or that it is expressed at levels insufficient to obtain full ε-toxin activation under the experimental conditions tested. Furthermore, here we report Western blot evidence for proteolytic processing of ε-toxin by lam^+ isolates, indicating that other *C. perfringens* proteases can also process ε-toxin. However, without trypsin treatment, little lethality was detected in the supernatants from the lam^+ isolates proteolytically processing ε-toxin (26), leaving the question whether these non-lambda toxin proteases can activate ε-toxin unresolved.

Finally, type B isolates also produce ε-toxin, although the virulence genetics of type B isolates have been investigated even less than those of type D isolates. Therefore, studies are now under way to characterize the virulence plasmids of type B isolates and to compare the results with our results obtained with type D isolates. The findings should provide further insight into the contribution of *C. perfringens* plasmids to virulence and the evolution of virulence plasmids.

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