

## Comparison of Virulence Plasmids among *Clostridium perfringens* Type E Isolates<sup>∇†</sup>

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*Clostridium perfringens* type E isolates produce iota-toxin, which is encoded by *iap* and *ibp* genes. Using Southern blot analyses, the current study identified *iap/ibp* plasmids of ~97 or ~135 kb among eight type E isolates. For most of these isolates, their *iap/ibp* plasmid also encoded urease and lambda-toxin. However, the beta2-toxin gene, if present, was on a different plasmid from the *iap/ibp* plasmid. For all isolates, the *iap/ibp* plasmid carried a *tcp* locus, strongly suggesting that these plasmids are conjugative. Overlapping PCR analyses demonstrated some similarity between the *iap/ibp* plasmids and enterotoxin-encoding plasmids of type A isolates. Additional PCR analyses demonstrated that the *iap/ibp* locus is located near *dcm* sequences, an apparent plasmid hot spot for toxin gene insertion, and that two *IS1151*-related sequences are present in the *iap/ibp* locus. To begin testing whether those *IS1151*-like sequences can mobilize *iap/ibp* genes, a PCR assay was performed that amplifies a product only from circular DNA forms that could represent transposition intermediates. This PCR assay detected circular forms containing *iap/ibp* genes and silent enterotoxin gene sequences, with or without an *IS1151*-like sequence. Collectively, these results suggest that a mobile genetic element carrying *iap/ibp* has inserted onto a *tcp*-carrying enterotoxin plasmid in a type A isolate, creating a progenitor *iap/ibp* plasmid. That plasmid then spread via conjugation to other isolates, converting them to type E. Further *iap/ibp* plasmid diversity occurred when either the *iap/ibp* genes later remobilized and inserted onto other conjugative plasmids or some *iap/ibp* plasmids acquired additional DNA sequences.

*Clostridium perfringens* isolates are classified into one of five types (A to E) (19, 25), based upon production of four typing toxins (alpha, beta, epsilon, and iota). Each *C. perfringens* type is associated with certain human or animal diseases (19, 25). Type E isolates cause enteritis or enterotoxemias in rabbits, lambs, cattle, and dogs (19).

By definition, type E isolates must produce both alpha- and iota-toxin (19, 25). Alpha-toxin is a 42.5-kDa single polypeptide with phospholipase C, sphingomyelinase, and hemolytic and lethal properties (23). Iota-toxin, a binary toxin comprised of two noncovalently lined components (named *ia* and *ib*), catalyzes ADP-ribosylation of host cell actin at Arg-177 (2). Beyond alpha- and iota-toxin, at least a few type E isolates have been shown to carry additional potential virulence genes encoding beta2-toxin, urease, or lambda-toxin (7, 12, 14, 16–18).

While the *plc* gene encoding alpha-toxin is chromosomal (22, 28), plasmids are also important for the virulence of *Clostridium perfringens* type E isolates. For example, it has been shown elsewhere (3, 12, 18) that many (if not all) type E isolates carry their *iap* and *ibp* genes (encoding, respectively, the *ia* activity or *ib* binding components of iota-toxin) on a large plasmid(s). While the *iap/ibp* plasmid remains poorly

characterized, it was found that highly conserved (but silent) *cpe* sequences are located immediately upstream of the plasmid-borne *iap/ibp* genes in many, if not all, type E isolates (3, 26).

The presence of highly conserved, silent *cpe* sequences in the *iap/ibp* locus of many or all type E isolates is interesting considering that *iap/ibp* homologues can also be carried by other pathogenic clostridial species, including *C. difficile* and *C. spiroforme* (15). The presence of similar binary toxin genes in other pathogenic clostridia led to the proposal (3) that a foreign genetic element carrying *iap/ibp* may have inserted into the promoter region of a plasmid-borne *cpe* gene in a type A isolate, creating a progenitor *iap/ibp* plasmid that subsequently transferred to other *C. perfringens* isolates, converting those plasmid recipients to type E. This hypothesis later received indirect support from a study (5) demonstrating that the *cpe* plasmids of type A isolates can transfer via conjugation, probably due to their carriage of a Tn916-related *tcp* locus similar to that mediating conjugative transfer of the *C. perfringens* tetracycline resistance plasmid pCW3 (1). Further supporting this hypothesis was the detection of *tcp* sequences in one type E isolate (21), although it was not determined whether *tcp* sequences are located on the *iap/ibp* plasmid in that or other type E isolates.

Support is also emerging for a close association between insertion sequences (ISs) and many *C. perfringens* toxin genes. For example, an *IS1469* sequence is located immediately upstream of the *cpe* gene in most or all type A isolates (6, 20). Furthermore, either *IS1151* or *IS1470*-like sequences are present downstream of most plasmid-borne *cpe* genes in type

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A isolates (20, 21). When chromosomal, the *IS1469-cpe* sequences are flanked by both upstream and downstream *IS1470* sequences that may represent an integrated transposon (6). It has been proposed that those flanking ISs can mobilize the chromosomal *cpe* gene since IS- and *cpe*-containing circular forms, which may represent transposition intermediates, have been detected in lysates of chromosomal *cpe* type A isolates (4). IS elements can also be associated with toxin genes in other *C. perfringens* types: e.g., an *IS1151* element is located upstream of the plasmid-borne *etx* gene of some type D isolates (11, 12). An *IS1151*-like sequence has also been identified downstream of the silent *cpe* sequences in many type E isolates (3), but it remains unknown whether any IS element is located downstream of the *iap/ibp* sequence in these isolates or whether IS elements can mediate movement of *iap/ibp*-containing DNA into circular intermediate forms.

Regardless of whether a downstream *IS1151* or *IS1470*-like sequence is present, the plasmid-borne *cpe* gene in type A isolates is always located near an upstream *dcm* gene (13, 20). That finding has suggested that the *dcm* region may represent a plasmid hot spot for insertion of mobile genetic elements carrying the *cpe* gene (21). Interestingly, similar *dcm* sequences have also been detected in some isolates of non-type A *C. perfringens*, including type E isolate 853 (20). However, it is unknown whether the *dcm* sequences of isolate 853 are located on the *iap/ibp* plasmid or whether *dcm* sequences are present in most type E isolates.

Because type E virulence plasmids have received such limited research attention to date, our current study sought to characterize their size, diversity, and virulence gene carriage using a previously studied collection of type E isolates mainly originating from diseased animals. In addition, to gain possible insights into *C. perfringens* virulence plasmid evolution, we further studied the organization of the *iap/ibp* locus in type E isolates in order to evaluate the possible presence in this locus of additional IS elements and to evaluate whether this locus is near *dcm* sequences.

#### MATERIALS AND METHODS

**Bacterial strains.** With the exception of type E reference strain NCIB10748 and isolate 576 (an NCIB10748 subculture obtained from J. Glenn Songer), the other six type E isolates used in this study, i.e., isolates 51, 294, 572, 853, 1987, and B2085, originated from neonatal calves that were diagnosed in the mid-1990s with hemorrhagic enteritis in the states of KS, MO, CO, and WY (3). Previous multiplex PCR analyses and iota-toxin activity assays (3) had classified these eight isolates as type E. By pulsed-field gel electrophoresis (PFGE), isolates NCIB10748, 51, 294, 572, 853, 1987, and B2085 did not appear to share a clonal relationship (3). All type E isolates were maintained as stock cultures in 15% glycerol and were stored at  $-80^{\circ}\text{C}$ .

Isolates ATCC 3624, strain 13, F5603, and F4969 are *C. perfringens* type A isolates. F5603 and F4969 carry, respectively, the previously sequenced *cpe* plasmid pCPF5603 or pCPF4969 (21). All of these type A isolates were maintained as stock cultures in cooked meat medium (Oxoid) and were stored at  $-20^{\circ}\text{C}$ . *Escherichia coli* strain DH5 $\alpha$  was grown in LB medium and used for transformation experiments.

**Preparation of DIG-labeled probes.** Digoxigenin (DIG)-labeled DNA probes were constructed using the PCR DIG probe synthesis kit (Roche). Primers for amplifying internal *iap*, *cpb2*, urease gene (*ure*), *cpe*, *tcpH*, *tcpF*, *IS1151*, *comEC*, and lambda-toxin gene (*lam*) sequences, as well as reaction 6 in the overlapping PCR assay for the pCPF4969 variable region (21), are listed in Table S1 in the supplemental material.

**PFGE and Southern hybridizations.** DNA plugs for PFGE were prepared as previously described (27). Briefly, overnight cultures of *C. perfringens* were grown in TGY (3% proteose peptone [Difco], 2% D-glucose [Fisher], 1% yeast extract

[Difco], and 0.1% sodium thioglycolate [Sigma]). Those cultures were centrifuged, and the bacterial pellets were resuspended in 2% PFGE-certified agarose (Bio-Rad), for a final agarose concentration of 1%. Those plugs were then electrophoresed in a CHEF-DR II PFGE system (Bio-Rad) maintained at  $14^{\circ}\text{C}$ , using 1% PFGE-certified agarose (Bio-Rad) and  $0.5\times$  Tris-buffered EDTA. The conditions used for PFGE, transfer of electrophoresed DNA to nylon membranes (Roche), and probe hybridization were as previously described (13). After probing, PFGE Southern blots were developed using reagents from the DIG DNA labeling and detection kit (Roche). When a blot was reprobed for the presence of additional genes, the blot was first stripped with 0.2 N NaOH and 0.1% sodium dodecyl sulfate and then incubated with prehybridization solution. Prior to hybridization with the second probe, the stripped blot was exposed to film to confirm that all signal had been removed during stripping. Probe hybridization was visualized with CSPD, a ready-to-use substrate from Roche.

**Restriction endonuclease digestion of pulsed-field gel plugs.** DNA plugs, prepared as described above, were washed carefully at room temperature with  $0.1\times$  Tris-EDTA buffer at least three times (15 min for each wash). A plug slice (corresponding to approximately one-sixth of the plug) was incubated overnight at proper temperature (as instructed by the manufacturer) in 30  $\mu\text{l}$  of  $10\times$  commercial restriction endonuclease buffer, 4  $\mu\text{l}$  of enzyme (NcoI, ClaI, StuI, BamHI, KpnI, PstI, SmaI, ApaI, BstXI, Sall, and XbaI; all from New England Biolabs), and 266 ml of distilled water. These digested plugs were then electrophoresed in a CHEF-DR II PFGE system using the following running conditions:  $14^{\circ}\text{C}$ , 6 V, and a 1- to 12-s pulse for 15 h.

**Overlapping PCR analyses to determine whether type E isolates carry the conserved or variable region of pCPF5603 or pCPF4969.** For these short-range PCRs, template DNA was obtained from *C. perfringens* colony lysates, as described previously (30). Each PCR mixture contained 2  $\mu\text{l}$  of template DNA, 17  $\mu\text{l}$  of TAQ Complete 1.1 X Master Mix (Gene Choice, Frederick, MD), and 1  $\mu\text{l}$  of each primer pair (1  $\mu\text{M}$  final concentration). Primers used for investigating whether type E isolates carry the variable region of pCPF5603 or pCPF4969 were listed previously (21). PCR conditions for these reactions were also described previously (21). Primers used for investigating whether type E isolates carry the conserved region of pCPF5603/pCPF4969 are listed in reference 21 and Table S2 in the supplemental material. Each PCR mixture contained 2  $\mu\text{l}$  of template DNA, 17  $\mu\text{l}$  of TAQ Complete 1.1 X Master Mix (Gene Choice, Frederick, MD), and 1  $\mu\text{l}$  of each primer pair (1  $\mu\text{M}$  final concentration). The reaction mixtures, with a total volume of 20  $\mu\text{l}$ , were placed in a thermal cycler (Techne) and subjected to the following amplification conditions: 1 cycle of  $95^{\circ}\text{C}$  for 2 min; 35 cycles of  $95^{\circ}\text{C}$  for 30 s,  $58^{\circ}\text{C}$  for 1 min, and  $72^{\circ}\text{C}$  for 1 min 40 s; and a single extension of  $72^{\circ}\text{C}$  for 10 min. PCR products were then electrophoresed on a 1% agarose gel, which was stained with ethidium bromide.

**Sequencing of the *iap/ibp* locus in type E isolates.** *C. perfringens* DNA was isolated from type E strain NCIB10748 using the MasterPure gram-positive DNA purification kit (Epicentre). The primers *dcm*-F (5'-TAATATTCCTTTCAGGCTTTC-3') and *iota*-R (5'-TAAATAAAACCTATAAACGC-3') were designed based upon previous results (3, 21). Those primers were added (at a 5  $\mu\text{M}$  final concentration) to a PCR mixture containing 5  $\mu\text{l}$  of purified NCIB10748 DNA template and Roche long-range PCR kit ingredients. The reaction mixtures, with a total volume of 50  $\mu\text{l}$ , were placed in a thermal cycler (Techne) and subjected to the following amplification conditions: 1 cycle of  $95^{\circ}\text{C}$  for 3 min; 40 cycles of  $95^{\circ}\text{C}$  for 30 s,  $55^{\circ}\text{C}$  for 1 min, and  $68^{\circ}\text{C}$  for 5 min; and a single extension of  $68^{\circ}\text{C}$  for 15 min. PCR products were then electrophoresed on a 1% agarose gel, which was stained with ethidium bromide. This PCR product was cloned into a TOPO vector (Invitrogen), which was sent for sequencing to the University of Pittsburgh Core Sequencing Facility.

**Overlap PCR analyses of the *iap/ibp* locus in type E isolates.** Primers overlapping the *iap/ibp* plasmid region spanning from *dcm* to the *IS1151*-like sequence downstream of the silent *cpe* sequences were designed based upon sequencing results obtained for this region (this study and reference 4; see also Table S3 in the supplemental material). Template DNA for these short-range PCRs was obtained from colony lysates, prepared as described previously (30). Each PCR mixture contained 2  $\mu\text{l}$  of template DNA, 17  $\mu\text{l}$  of TAQ Complete 1.1 X Master Mix (Gene Choice, Frederick, MD), and 1  $\mu\text{l}$  of each primer pair (1  $\mu\text{M}$  final concentration). PCR amplification was then performed in a Techne (Burkhardtendorf, Germany) thermocycler using the following conditions:  $94^{\circ}\text{C}$  for 2 min; 35 cycles of  $94^{\circ}\text{C}$  for 30 s,  $54^{\circ}\text{C}$  for 1 min, and  $72^{\circ}\text{C}$  for 1 min 30 s; and a single extension of  $72^{\circ}\text{C}$  for 10 min. PCR products were separated on 1% agarose gels and visualized with ethidium bromide staining.

**PCR evaluation of *iap/ibp*-carrying circular intermediate formation.** Each PCR mixture contained 5  $\mu\text{l}$  of template DNA (freshly prepared lysate from an overnight brain heart infusion culture of isolate 853), 44  $\mu\text{l}$  of TAQ Complete 1.1 X Master Mix (Gene Choice, Frederick, MD), and 1  $\mu\text{l}$  of each primer pair (1

$\mu$ M final primer concentration). Primers used in these studies included P1-R and P8-F from Table S3 in the supplemental material and L1-F (5'-TGTATCCAGTGATATTATTAC-3') and L1-R (5'-TTAGAAGAATATAAGAAAGAAC-3'). The PCRs that were run included reaction R1 (using primers L1-R and P8-F), R2 (using primers L1-R and L1-F), R3 (using primers P1-R and L1-F), and R4 (using primers P1-R and P8-F). PCR amplification was then performed in a Techne thermocycler using the following conditions: 94°C for 2 min; 35 cycles of 94°C for 30 s, 54°C for 30 s, and 72°C for 2 min 30 s; and a single extension of 72°C for 15 min. PCR products were separated on 1.5% agarose gels and visualized with ethidium bromide staining. PCR products were also excised from the gel, ligated into a Topo vector, and sequenced.

**Nucleotide sequence accession number.** The sequencing results were deposited in GenBank under accession number EF179380.

## RESULTS

**Pulsed-field gel Southern blot examination of *iap*-carrying plasmids in type E isolates.** Previous studies by several laboratories (3, 8, 10, 12, 13, 18, 21) have demonstrated that Southern blot assays of pulsed-field gels provide useful insights into the size, gene carriage, and diversity of *C. perfringens* virulence plasmids. Therefore, this technique was applied to a collection of type E isolates in order to (i) evaluate whether selected genes are commonly present among type E isolates; (ii) determine whether those genes, when present, are located on the chromosome or on plasmids; and (iii) evaluate the size and diversity of type E virulence plasmids.

Our initial pulsed-field Southern blot analyses focused on the *iap/ibp* plasmid, whose presence is required for the iota-toxin production that defines type E isolates. For this experiment, undigested DNA from eight type E isolates was subjected to PFGE under conditions allowing undigested plasmid DNA (but not undigested chromosomal DNA) to enter the pulsed-field gels (3, 8, 10, 12, 13, 18, 21). The result of Southern blot assays demonstrated (Fig. 1A), for all eight type E isolates, that an *iap*-specific probe hybridized with undigested DNA that had entered the pulsed-field gels, confirming previous studies (3) showing that the *iap* gene is plasmid borne in all these type E isolates. As a negative control, the *iap* probe did not hybridize with DNA from type A isolate F5603, which is *iap* negative by PCR (data not shown).

It has also been established previously that, on these pulsed-field Southern blots, probes hybridize predominately to the linearized plasmid form, thus allowing an accurate determination of plasmid size (10, 12, 13, 18, 21). This approach had previously shown that the *iap/ibp* plasmid in NCIB10748 is ~130 kb in size (18), but the size of the *iap/ibp* plasmid in other type E isolates had not been evaluated prior to our current study. As shown in Fig. 1A, this Southern blot revealed the existence of size variations among *iap/ibp* plasmids of different type E isolates. Specifically, in six of the eight type E isolates surveyed, the *iap/ibp* virulence plasmid was ~135 kb, while the other two type E plasmids were found to carry an *iap/ibp* plasmid of only ~97 kb. It is noteworthy that the ~135-kb size determined in the current study for the *iap/ibp* plasmid of NCIB10748 closely matched the previous ~130-kb size estimate for this plasmid (18).

Previous Western blotting and DNA sequencing results (3) demonstrated that the eight type E isolates surveyed each carry silent *cpe* sequences near their *iap/ibp* genes. When the Fig. 1A Southern blot was stripped and rehybridized with a *cpe* probe, the results obtained were consistent with those previous find-

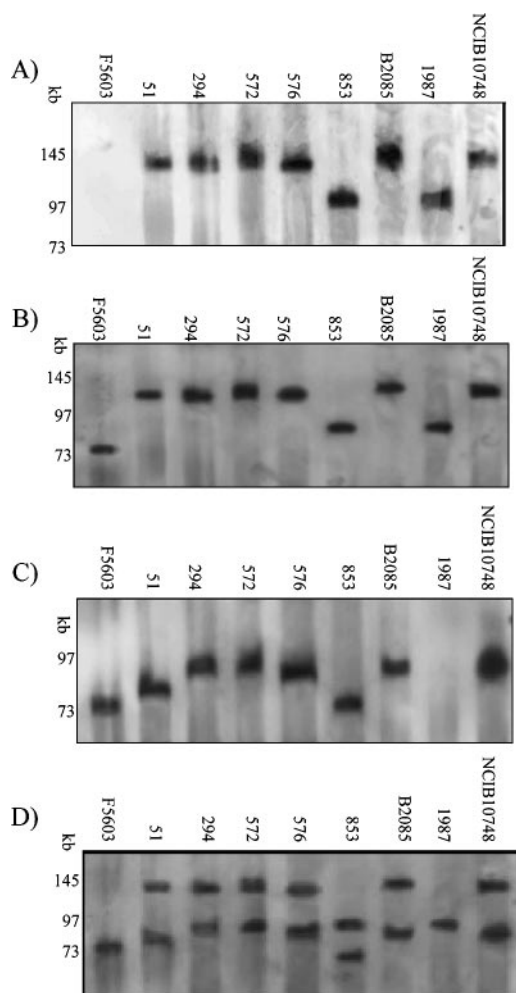


FIG. 1. Southern blot of a pulsed-field gel electrophoresed with DNA from specified type E isolates and then hybridized with an *iap* probe (A). After stripping, the blot was rehybridized with a *cpe* probe (B). After a final stripping, the blot was rehybridized with a *cpb2* probe (C). An overlay of the panel B and panel C blots demonstrates that *cpb2* is located on a different plasmid than the iota-toxin locus (D). Numbers at left of each blot indicate migration of size markers in kb.

ings, i.e., for each of these eight type E isolates, a hybridization signal was observed at the same blot location containing the *iap/ibp* plasmid (Fig. 1B). The specificity of this *cpe* probe hybridization with DNA from the type E isolates (and *cpe*-positive type A isolate F5603) was supported by the failure of the probe to react with DNA from *cpe*-negative type A isolate ATCC 3624 or strain 13 (data not shown).

**Pulsed-field gel Southern blot analyses of type E isolates to assess their plasmid carriage of *cpb2*, *lam*, *ure*, IS1151-like sequences, and *tcp* conjugative transfer genes.** Other laboratories have reported (17) that type E isolates often carry the *cpb2* gene encoding beta2-toxin, a potential accessory toxin for virulence. However, to our knowledge, it had not yet been determined whether the *cpb2* gene in type E isolates is plasmid borne and, if so, whether this toxin gene is present on the *iap* plasmid. To address those questions, the Fig. 1B Southern blot was stripped and reprobbed with a *cpb2*-specific probe. The

TABLE 1. Sizes of virulence plasmids among type E isolates

Strain	Type	Size(s) (kb) of plasmid(s) carrying gene or element:							
		<i>iap/ibp</i>	<i>cpe</i>	<i>ure</i>	<i>lam</i>	<i>cpb2</i>	<i>tcpH</i>	<i>tcpF</i>	IS1151
F5603	A		75			75	75	75	75
51	E	135	135	135	135	85	135, 65	135, 65	135, 85
294	E	135	135	135	135	97	135	135	135, 97
572	E	135	135	135	135	97	135, 65	135, 65	135, 97
576	E	135	135	135, 90	135	90	135, 90	135, 90	135, 90
853	E	97	97	97	97	70	97, 70	97, 70	97, 70
1987	E	97	97		97		97	97	97
B2085	E	135	135	135	135	90	135, 65	135, 65	135, 90
10748 <sup>a</sup>	E	135	135	135	135	90	135, 65	135, 65	135, 90

<sup>a</sup> NCIB10748.

results obtained (Fig. 1C and Table 1) demonstrate that, in seven of the eight type E isolates surveyed, the *cpb2* gene is present on plasmids that range in size from ~70 to ~97 kb. The single type E isolate (1987) whose DNA failed to hybridize with the *cpb2* probe on this Southern blot also tested *cpb2* negative by PCR (data not shown). In contrast to the limited diversity of *iap/ibp* plasmid sizes detected among the surveyed type E isolates in Fig. 1A, five differently sized *cpb2*-carrying plasmids were demonstrated among the seven *cpb2*-positive type E isolates. Furthermore, each of the seven *cpb2*-positive type E isolates clearly carried its *cpb2* gene on a plasmid distinct from the *iap/ibp* plasmid. As specificity controls, the *cpb2* probe was shown to hybridize with F5603 DNA (Fig. 1C), which carries *cpb2* on pCPF5603 (21), but failed to hybridize with DNA from *cpb2*-negative isolate ATCC 3624 (data not shown).

Two other potential virulence genes, i.e., *ure* and *lam*, have been detected in one or a few type E isolates (12, 14, 16, 18); in addition, *ure* genes were shown to be plasmid borne in two type E isolates, including NCIB10748 (12). However, it had not yet been systematically investigated whether *ure* and *lam* are commonly present in type E isolates and, if so, whether they are present on the *iap/ibp* plasmid (although one study [12] suggested that *ure* might be on the *iap/ibp* plasmid of NCIB10748). Therefore, pulsed-field gel Southern blot assays were performed using *lam* or *ure* probes, which demonstrated (Table 1) the presence of *lam* in all eight type E isolates surveyed. Furthermore, for each type E isolate surveyed, the *lam* probe hybridized to the same blot location containing the *iap/ibp* plasmid. In contrast, DNA from one type E isolate (1987) failed to hybridize to the *ure* probe (Table 1); isolate 1987 was also PCR negative for *ure*, while the other seven isolates were PCR positive (data not shown). For the seven *ure*-positive type E isolates, the *ure* probe consistently hybridized to the same blot location containing the *iap/ibp* plasmid. In addition, type E isolate 576 was also found to carry a second, smaller *ure* plasmid (Table 1). As a negative control for probe specificity, the *ure* and *lam* probes each failed to hybridize with DNA from F5603, which is *ure* negative and *lam* negative by PCR (data not shown).

Our previous sequencing studies (3) had demonstrated the presence of an IS1151-like sequence ~1 kb downstream of the silent *cpe* sequences in the *iap/ibp* locus of these eight surveyed type E isolates. Consistent with that previous finding, all eight type E isolates hybridized to an IS1151-like specific probe to

the same blot location containing the *iap/ibp* plasmid. For seven of these type E isolates (the exception being 1987, the only *cpb2*-negative type E isolate surveyed), the IS1151-like probe also hybridized to a second plasmid. The additional IS1151-like carrying plasmids, which ranged in size from 70 to 97 kb, consistently matched the size of the *cpb2* plasmid present in each of those seven isolates (Table 1).

The conjugative mechanism for *C. perfringens* plasmid pCW3 (and probably pCPF4969 and pCPF5603 [21]) was recently shown (1) to involve a Tn916-related *tcp* locus, and similar *tcp* genes had been previously detected in one type E isolate (21). Since we previously hypothesized that *iap/ibp* plasmids are conjugative (3), Southern blot analyses of pulsed-field gels were performed to evaluate whether *tcp* conjugative transfer genes are commonly present in type E isolates and, if so, whether they are associated with the *iap/ibp* plasmid in our eight surveyed type E isolates. Using probes for two different *tcp* genes, each known to be essential for pCW3 plasmid conjugation (1), hybridization was observed with DNA from all eight type E isolates (Table 1). Furthermore, the *tcpH* and *tcpF* probes hybridized to the same blot location containing the *iap/ibp* plasmid for each of the type E isolates surveyed. In addition, both the *tcpH* and *tcpF* probes also hybridized to a second plasmid in six of the eight type E isolates. For five of those six type E isolates, their second *tcpH*- and *tcpF*-carrying plasmid was clearly distinct from the *cpb2* plasmid. However, for isolate 576, the *tcpH*, *tcpF*, and *cpb2* probes all hybridized to a plasmid(s) of ~90 kb.

As controls for *tcpH* and *tcpF* probe specificity, similar Southern blot assays of pulsed-field gels demonstrated that (i) both *tcp* probes hybridized to pCPF5603, which has been shown by sequencing to carry *tcpH* and *tcpF* (21), but (ii) both *tcp* gene probes failed to hybridize with DNA from strain 13, whose single plasmid is devoid of the *tcp* locus (28).

**Pulsed-field gel Southern blot analysis of restriction endonuclease-digested DNA to distinguish whether comigrating genes in type E isolates are present on the same plasmid or two similarly sized plasmids.** The pulsed-field gel Southern blotting results presented in Fig. 1 and Table 1 could indicate either that (i) *ure*, *lam*, *tcpF*, *tcpH*, and IS1151-like are often found on the *iap/ibp*-carrying plasmid or (ii) in type E isolates, those genes are often present on plasmids that share a similar size but are distinct from *iap/ibp* plasmids. To help discriminate between those two possibilities, DNA from representative type E isolates 853 and NCIB10748 was subjected to digestion with

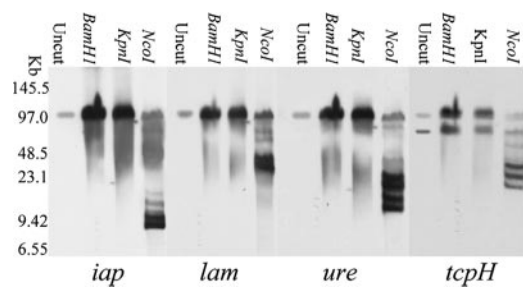


FIG. 2. Southern blot of a pulsed-field gel electrophoresed with DNA from type E isolate 853 and then digested with specified restriction enzymes for 16 h. The Southern blot was first hybridized with an *iap* probe and then sequentially stripped and rehybridized with *lam*, *ure*, and *tcpH* probes.

a restriction enzyme battery. In this experiment (10), the migration of two genes present on the same plasmid should consistently exhibit the same response (sensitivity or insensitivity) to digestion with each restriction endonuclease. However, if one gene showed no change in migration after digestion with a particular restriction endonuclease while migration of the second gene was affected by the same enzyme, this would indicate that the two genes are present on similarly sized, but distinct, plasmids.

When this analysis was performed with the representative type E isolates 853 and NCIB10748, the results obtained supported the presence of *lam*, *ure*, *tcpH*, and *tcpJ* on the *iap/ibp*-carrying plasmid of both isolates (Fig. 2 and data not shown). Specifically, the migration of plasmid DNA carrying *iap*, *lam*, *ure*, *tcpH*, and *tcpJ* was affected by NcoI, ClaI, and StuI but not by BamHI, KpnI, PstI, SmaI, ApaI, BstXI, SalI, or XbaI.

**PCR analyses to determine whether the pCPF4969/pCPF5603 conserved region is present among type E isolates.** An internal

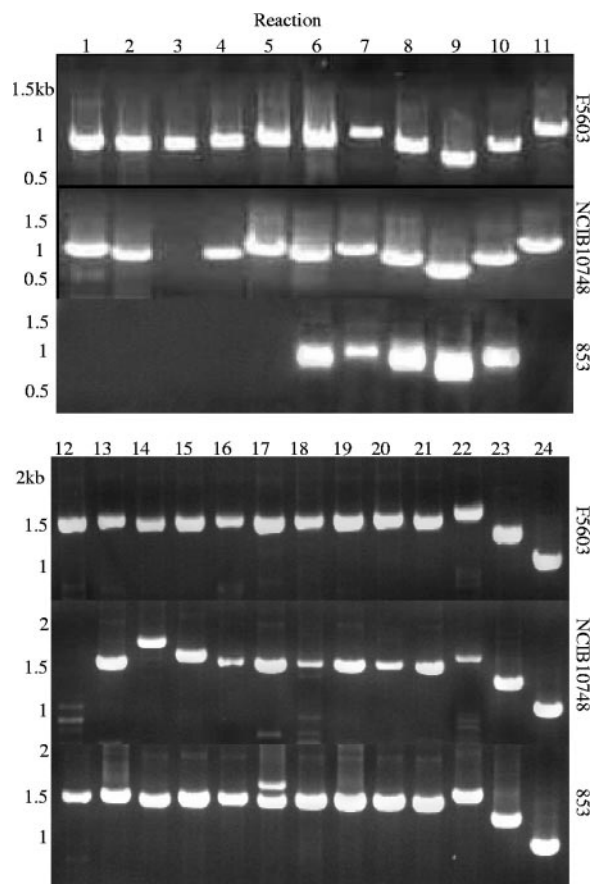


FIG. 3. Overlapping PCR amplification of the conserved region of pCPF5603/pCPF4969 using a previously described primer battery (21) and one described in this study (see Table S2 in the supplemental material). DNA template included F5603 DNA (top), NCIB10748 DNA (middle), and 853 DNA (bottom). Marker sizes are shown at left.

TABLE 2. Results for individual ORF PCRs<sup>a</sup>

Gene	F5603	853	1987	51	572	B2085	NCIB10748	294	576
Conserved region									
<i>dcm</i>	+	+	+	+	+	+	+	+	+
<i>cpe</i>	+	+	+	+	+	+	+	+	+
<i>IS1151</i>	+	+	+	+	+	+	+	+	+
<i>ftsK</i>	+	+	+	+	+	+	+	+	+
<i>tcpC</i>	+	+	+	+	+	+	+	+	+
<i>tcpH</i>	+	+	+	+	+	+	+	+	+
<i>tcpF</i>	+	+	+	+	+	+	+	+	+
<i>dam</i>	+	+	+	+	+	+	+	+	+
<i>cna</i>	+	+	+	+	+	+	+	+	+
<i>srt</i>	+	+	+	+	+	+	+	+	+
<i>lexA-1</i>	+	+	+	+	+	+	+	+	+
<i>pspC</i>	+	+	+	+	+	+	+	+	-
<i>hel</i>	+	+	+	+	+	+	+	+	-
<i>flgJ</i>	+	+	-	-	+	+	+	-	-
Variable region									
<i>capK</i>	+	+	+	+	+	+	+	+	-
<i>pri</i>	+	+	-	+	-	-	-	-	-
<i>comEC</i>	+	+	+	+	-	-	-	-	-
<i>his</i>	+	+	-	-	-	-	-	-	-
<i>thiF</i>	+	+	-	-	-	-	-	-	-
<i>cpb2</i>	+	+	-	+	+	+	+	+	+
<i>ure</i>	+	+	-	+	+	+	+	+	+

<sup>a</sup> +, present; -, absent.

open reading frame (ORF)-specific PCR analysis showed (Table 2) that either all (isolate 853) or most (the remaining seven type E isolates) of 14 surveyed ORFs in the conserved region of pCPF4969/pCPF5603 are also present in the eight surveyed type E isolates. Notably, these analyses confirmed the pulsed-field Southern blotting results indicating that all eight type E isolates carry the *tcp* ORFs known to mediate conjugative transfer of *C. perfringens* plasmid pCW3 and (probably) pCPF4969 and pCPF5603.

Overlapping PCR analyses were then performed to assess the arrangement of those conserved-region ORFs in the surveyed type E isolates; this 24-reaction analysis, which spans from an *IS1151* located near *cpe* to the start of the pCPF5603 or pCPF4969 variable region, revealed (Fig. 3; see also Table S4 in the supplemental material) that all eight isolates carry a *tcp* region arranged similarly to that present on pCPF5603, rather than the slightly different *tcp* locus arrangement of pCPF4969. This assay also clearly demonstrated (Fig. 3 and data not shown) that one type E isolate (NCIB10748) carries virtually the entire pCPF4969/pCPF5603 conserved region, with the remaining seven isolates carrying most of the pCPF4969/pCPF5603 conserved region.

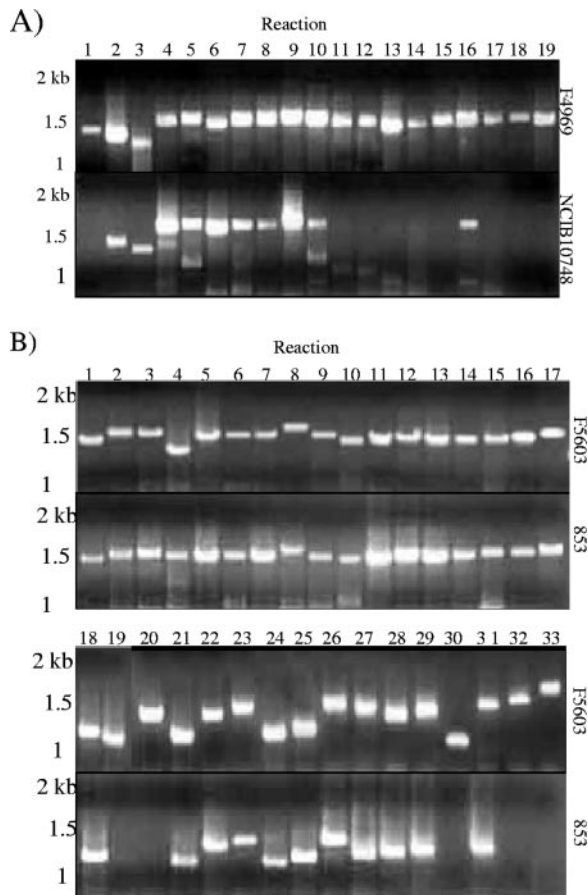


FIG. 4. Overlapping PCR amplification of the pCPF4969 (A) or pCPF5603 (B) variable region using DNA from type E isolates NCIB10748 and 853. No substantial amplification was observed using DNA from the other six surveyed type E isolates. Marker sizes are shown at left.

**PCR analyses to determine whether the pCPF4969 or pCPF5603 variable region is present among type E isolates.** The presence of the pCPF4969 variable region in the eight type E isolates was evaluated using a previously described 24-reaction, overlapping PCR assay that spans the entire pCPF4969 variable region (21). This PCR battery amplified only two of the expected 24 PCR products from seven type E isolates (see Table S5 in the supplemental material). However, this assay indicated that NCIB10748 apparently carries a more substantial portion (from reaction 2 to reaction 10) of the pCPF4969 variable region (Fig. 4A). A Southern blot assay of a pulsed-field gel with a probe to reaction 6 of the pCPF4969 variable-region overlapping PCR assay confirmed that pCPF4969 variable-region sequences are present on the *iap/ibp* plasmid of NCIB10748.

A second, 33-reaction overlapping PCR battery spanning the entire pCPF5603 variable region (21) was then performed to survey the presence of the pCPF5603 variable region in the surveyed type E isolates. With this PCR battery, type E isolate 853 showed significant amplification over a substantial portion of the pCPF5603 variable region (Fig. 4B; see also Table S6 in the supplemental material), while isolate 1987 supported amplification of reactions 21 to 29 of this assay. The other six

isolates amplified only a few products (never representing more than three adjacent sequence reactions) in this assay. To assess whether the amplified pCPF5603 reactions of isolates 853 and 1987 were present on the *iap/ibp* plasmid, a pulsed-field gel was run with DNA from those two isolates and hybridized with a probe to the putative *comEC* ORF of the pCPF5603 variable region. That probe failed to react with NCIB10748 DNA, consistent with Table 2 results, but hybridized to a plasmid of about 97 kb in isolates 1987 and 853, matching the size of the *iap/ibp* plasmid in both those isolates.

**Further characterization of the *iap/ibp* locus in type E isolates.** The *iap/ibp* locus, previously shown to be highly conserved among these eight type E isolates (3), consists of an *IS1151*-like sequence (now referred to as *IS1151-2*) upstream of silent *cpe* sequences, along with the iota-toxin genes and silent *cpe* sequences (Fig. 5A). Since the plasmid-borne *cpe* gene in both pCPF4969 and pCPF5603 is present near *dcm* sequences (13, 21), long-range PCR analyses were performed to evaluate whether the *iap/ibp* genes of type E isolates might also be located near a *dcm* gene. Those long-range PCR assays successfully linked *dcm* to the *iap* gene for all eight surveyed type E isolates (data not shown). This reaction produced a 4.3-kb PCR product that, when sequenced from NCIB10748, revealed that the *iap/ibp* locus also contains a second, partial copy of *IS1151* (now termed *IS1151-1*) located between *dcm* and *iap*, as shown in Fig. 5A.

This new information demonstrating that the *iap/ibp* locus is flanked by *IS1151*-like sequences could indicate that the *iap/ibp* genes are present on an integrated transposon. To start addressing whether the *iap/ibp* locus of type E isolates can be mobilized, we employed PCR methods similar to those used previously (4) to demonstrate that the chromosomal *cpe* locus of type A isolates can form circular species that could represent transposition intermediates. Specifically, these PCRs employed primers that are unable to amplify a product from a linear *iap/ibp* locus but yield PCR products if this region can form circular intermediates. Those analyses successfully amplified several PCR products from all eight type E isolates (Fig. 5B). When two of those products (from isolate 853) were sequenced, they corresponded to circular products containing either *iap-ibp*-silent *cpe* sequences or *iap-ibp*-silent *cpe*-*IS1151-2* sequences (Fig. 5C).

## DISCUSSION

Despite the significance of type E isolates as veterinary pathogens, their virulence plasmids had received only limited research attention. In the current study, Southern blot assays of pulsed-field gels revealed previously unappreciated size, sequence, or arrangement variations among the iota-toxin-encoding plasmids of type E isolates. Most surveyed type E isolates were found to carry their *iap* genes (and, by extension, *ibp* genes) on ~135-kb plasmids, which represent the largest virulence plasmids identified, to date, in *C. perfringens*. However, precedent exists for even larger virulence plasmids in other gram-positive bacteria: e.g., pXO1 of *Bacillus anthracis* is ~182 kb (24). Our results also identified variations in gene sequence or arrangement, even among *iap/ibp* plasmids of similar size. For example, an overlapping PCR assay amplified some pCPF4969 variable-region sequences from NCIB10748 but not

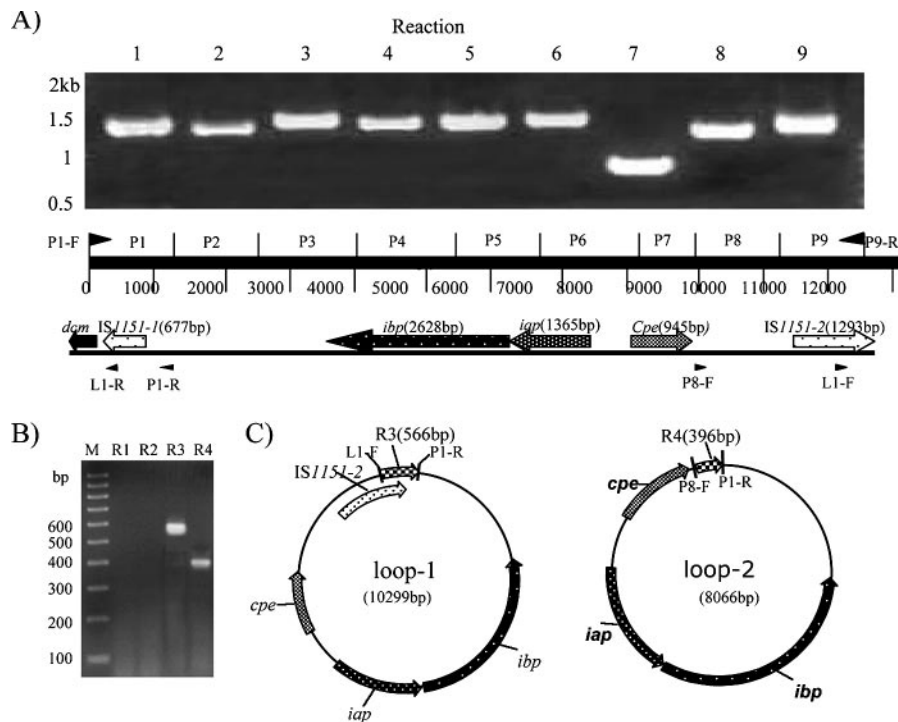


FIG. 5. Further characterization of the *iap/ibp* locus arrangement and demonstration that this locus can exist as circular intermediates that could be transposition intermediates. Based upon sequencing of a long-range PCR product connecting *dcm* to the *iap/ibp* locus in NCIB10748 (not shown), an overlapping PCR assay was designed and applied to the eight surveyed type E isolates. Results for NCIB10748 are shown in panel A; similar results were also observed with the other seven isolates. Panel B shows results from a PCR assay that used primer sets that amplify products from the *iap/ibp* locus of NCIB10748 when this locus is present in a small circular form but not in a linear form. The primer sets used are R1 (primers L1-R and P8-F), R2 (L1-R and L1-F), R3 (P1-R and L1-F), and R4 (P1-R and P8-F); locations of primers are shown in panel A. Panel C shows the expected products from small circular forms containing *IS1151-2-ibp-iap-silent cpe* sequences or *ibp-iap-silent cpe* sequences. The identity of these products was confirmed by DNA sequencing.

from the other type E isolates carrying an ~135-kb *iap/ibp* plasmid.

Results from this study have also clearly established that *iap/ibp* plasmids often, although not universally (this study and reference 15), carry *lam* and *ure* as additional potential virulence genes. The common presence of *lam* on *iap/ibp* plasmids is interesting since the *ua* and *tb* components of iota-toxin must be proteolytically activated to gain toxicity (2) and lambda-toxin is a metalloprotease known to activate iota-toxin (15). Therefore, the presence of *lam* on many *iap* plasmids may ensure that those type E strains can activate their iota-toxin. Urease also could contribute to *C. perfringens* virulence, since this enzyme is a proven virulence factor for other gastrointestinal pathogens (9). Conversely, urease conceivably might inhibit type E isolate virulence by raising the microenvironment pH, thus interfering with internalization of *ua* into the host cell cytoplasm, since that process involves endosomal acidification (2). These alternatives should be resolved by future studies.

Our results confirmed previous surveys (17) indicating that many type E isolates carry the *cpb2* gene, although we also identified a *cpb2*-negative type E isolate. Interestingly, Southern blots clearly showed that, when present, the *cpb2* gene in type E isolates is present on a different plasmid than that carrying the *iap/ibp*, *ure*, and *lam* genes, i.e., many type E isolates maintain at least two distinct potential virulence plasmids. In that respect, type E isolates resemble those type A

isolates carrying pCPF4969-like *cpe* plasmids and a second, distinct *cpb2* plasmid (13). A novel aspect of the type E *cpb2* plasmids is their large size, which extends up to ~97 kb; in contrast, the largest identified *cpb2*-carrying plasmids of type A isolates are only 75 kb (13). Further studies should examine the relationship between the *cpb2* plasmids of different type E isolates, their relationship to the *cpb2* plasmids of type A isolates, and how commonly the large type E *cpb2* plasmids encode additional virulence factors. However, our results already suggest that the *cpb2* plasmid of isolate 576 carries a second potential virulence gene, i.e., *ure*. Interestingly, the presence of this *ure*<sup>+</sup> *cpb2*<sup>+</sup> plasmid in isolate 576 represents only one of several differences detected in our study between that isolate and NCIB10748, which should be the same isolate supplied from different lab sources. PFGE gels comparing MluI- or ApaI-digested DNA from NCIB10748 and 576 (not shown) suggested that these isolates are closely related but also revealed some minor banding differences. Collectively, these results suggest that 576 and NCIB10748 share a common origin but now exhibit some differences among their plasmids.

Our current results demonstrate that many *iap/ibp* plasmids carry a *tcp* locus similar to that present on known *C. perfringens* conjugative plasmids (21), predicting that these *iap/ibp* plasmids should also be conjugative. The current study also found that many type E isolates possess more than one *tcp*-carrying plasmid, suggesting that a single *C. perfringens* cell can carry

multiple conjugative plasmids. For example, the *cpb2* plasmid of isolate 853 also may be conjugative, marking the first identification of a potential conjugative plasmid carrying *cpb2* in the absence of *cpe* or silent *cpe* sequences.

Results from the current and previous (3) studies indicate that the *iap/ibp* locus in many type E isolates is flanked by upstream and downstream *IS1151*-related sequences. This arrangement could indicate either that (i) the *iap/ibp* genes (and silent *cpe* sequences) are part of an integrated composite transposon that had flanking *IS1151*-like sequences or that (ii) an element containing *IS1151-iap-ibp* inserted near the *cpe* promoter on a pCPF5603-like plasmid (21), where *IS1151*-like sequences were already present downstream of the *cpe* gene. Interestingly, another laboratory reported that type E isolate CN5056 has an *IS1469*, rather than *IS1151*, sequence downstream of its silent *cpe* sequences in the *iap/ibp* locus (26). In our current study, PCR indicated (not shown) that at least six of the eight surveyed type E isolates do not carry *IS1469*. Further surveys should determine whether CN5056 is a unique type E isolate or represents a second, common lineage of type E isolates. Our current results further suggest that *IS1151*-related sequences are also present on the *cpb2* plasmid in most type E isolates. Future studies should determine if those *IS1151*-like sequences are associated with *cpb2*, which could help explain the broad distribution of the *cpb2* gene among *C. perfringens* isolates.

Our initial model for type E isolate evolution proposed that a mobile genetic element carrying *iap/ibp* had inserted near the promoter of a plasmid-borne *cpe* gene in a type A isolate, inactivating that *cpe* gene and permitting the accumulation of additional mutations. To account for the presence of highly conserved mutations in the silent *cpe* sequences of our eight chromosomally distinct type E isolates, we further proposed that this progenitor *iap/ibp* plasmid had then conjugatively transferred to a number of other type A isolates, converting those isolates to type E. The current results support certain aspects of our previous model. For example, our PCR results showed that all of the surveyed type E *iap/ibp* plasmids, but particularly the ~97-kb *iap/ibp* plasmids of 853 and 1987, share some sequence similarity with the *cpe* plasmids of type A isolates. The current study also found that these *iap/ibp* plasmids typically carry a *tcp* locus, which predicts that these plasmids should be conjugative (1). Finally, supporting the model's suggestion that a mobile genetic element carrying the *iap/ibp* genes had inserted into a *cpe* plasmid of a type A isolate, our current results revealed that (i) *iap/ibp* genes can be found in circular forms that could be transposition intermediates and (ii) a downstream, partial *IS1151*-like sequence is present that could once have helped mediate *iap/ibp* insertion into a *cpe* plasmid. Interestingly, we found that those *IS1151-1-iap-ibp* sequences are present near *dcm* sequences in type E isolates, further supporting the *dcm* region as an apparent hot spot for toxin gene insertion onto *C. perfringens* plasmids (21).

However, our current results also revealed some inconsistencies with our previous model for type E isolate evolution. Most notably, that model had predicted that *iap/ibp* plasmids should all be similar, yet our current studies demonstrated substantial size and sequence or arrangement variations among type E *iap/ibp* plasmids. One possible explanation for this discrepancy vis-à-vis our original model would be for the

~97-kb *iap/ibp* plasmids to represent a progenitor *iap* plasmid, arising from the 75-kb pCPF5603 *cpe* plasmid acquiring an *iap/ibp*-carrying mobile genetic element. Whether that element also carried *lam* and *ure* or whether those (and other) genes were acquired later in independent insertional events remains to be determined. In this scenario, the ~135-kb *iap/ibp* plasmids present in the other six surveyed type E isolates might simply represent an ~97-kb *iap/ibp* plasmid that was further enlarged by acquiring additional DNA sequences. If so, some of the original pCPF5603 DNA was lost or rearranged during this enlargement process, since the ~135-kb *iap/ibp* plasmids lack nearly all of the pCPF5603 variable-region genes found in the ~97-kb *iap/ibp* plasmids. An alternative model is that the *iap/ibp* locus later remobilized from the ~97-kb plasmid and then reinserted onto another *C. perfringens* plasmid or plasmids to give rise to the ~135-kb *iap* plasmids. This second possibility is supported by the presence of multiple large *tcp*-carrying plasmids in some type E isolates that could be targets for *iap/ibp* element insertion. However, this model would not readily explain why both the ~97-kb and ~135-kb *iap* plasmids share some *cpe* plasmid sequences or carry *ure* and *lam*, unless those two genes had comobilized with the *iap/ibp* genes. In any event, it is notable that all large *C. perfringens* plasmids studied to date (this study; 1, 3, 21, 28), whether toxin or antibiotic resistance encoding, carry a similarly arranged region containing *cna*, *srt*, and *dam* ORFs, suggesting a common evolutionary core for many large *C. perfringens* plasmids. Sequencing of several type E plasmids would help to resolve the remaining questions regarding the evolution of *iap* plasmids among different type E strains and resolve whether the very large *iap* plasmids carry additional, still-unrecognized, virulence genes.

As mentioned in the introduction, *iap/ibp*-like genes are also found in *C. spiroforme* and *C. difficile* (2), including the newly emergent *C. difficile* epidemic strains (29). In fact, our type E evolution model had proposed that type E isolates originally acquired their *iap/ibp* genes from another clostridial species. This possibility is supported by our results suggesting that the *iap/ibp* genes are associated with mobilizable genetic elements. This apparent association of *iap/ibp* genes with mobilizable elements may also help to explain the distribution of *iap/ibp*-like genes among several clostridial species. For example, two clostridial species in the gastrointestinal tract or soil may have exchanged a conjugative plasmid carrying an *iap/ibp*-like genetic element, which could then have mobilized and stably inserted onto the chromosome or another plasmid in the recipient clostridial species. Future studies should compare the *iap/ibp*-like genes found in other clostridial species with those found in *C. perfringens* type E isolates to gain further insights into clostridial virulence evolution.

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