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SHORT COMMUNICATION

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# Genome mapping of *Clostridium perfringens* strains with I-*Ceu*I shows many virulence genes to be plasmid-borne

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**Abstract** The intron-encoded endonuclease I-*Ceu*I from *Chlamydomonas eugametos* was shown to cleave the circular chromosomes of all *Clostridium perfringens* strains examined at single sites in the rRNA operons, thereby generating ten fragments suitable for the rapid mapping of virulence genes by pulsed-field gel electrophoresis (PFGE). This method easily distinguishes between plasmid and chromosomal localisations, as I-*Ceu*I only cuts chromosomal DNA. Using this approach, the genes for three of the four typing toxins, $\beta$, $\varepsilon$, and $\iota$, in addition to the enterotoxin and $\lambda$-toxin genes, were shown to be plasmid-borne. In a minority of strains, associated with food poisoning, where the enterotoxin toxin gene was located on the chromosome, genes for two of the minor toxins, $\theta$ and $\mu$, were missing.

**Key words** Gangrene · Toxins · Enterotoxin · Plasmids · Genome mapping

## Introduction

The Gram-positive anaerobe *Clostridium perfringens* causes several serious diseases in humans and domesticated livestock (Rood and Cole 1991). It is the principal agent of gas gangrene and is a common cause of human food poisoning (Granum 1990; McDonel 1986; Rood and Cole 1991). Clinical isolates are classified into five serotypes, A to E, according to their secretion of four major exotoxins, $\alpha$-, $\beta$-, $\varepsilon$-, and $\iota$-toxin, and they also produce a number of minor exotoxins involved in pathogenesis as indicated in Table 1 (for reviews see Hatheway 1990; Rood and Cole 1991).

A detailed physical map of the chromosome of the well-characterised type A strain of *C. perfringens*, CPN50, is available and the locations of several virulence genes (Fig. 1) are known (Canard and Cole 1989; Katayama et al. 1995). Comparative mapping of seven strains, including some clinical isolates, demonstrated that although the structure and organisation of most of the chromosome were conserved, five virulence genes (*colA*, *cpe*, *nagH*, *pfoA*, and *plc*) were associated with variable regions (Canard et al. 1992; Katayama et al. 1995). In contrast, the *nanH* gene was localized in a conserved region (Canard et al. 1992), while *etx* was located on plasmids (Canard et al. 1992; Cornillot et al. 1995).

Recently, it was shown that the enterotoxin (CPE) gene, *cpe*, which was situated in variable region **c** of strain 8–6, may be located either on the chromosome or on a plasmid (Cornillot et al. 1995) and that strains with chromosomal *cpe* genes were rarer but more likely to be involved in human food poisoning. This raised the question of whether the other virulence genes in variable regions (*colA*, *nagH*, *pfoA*, and *plc*) were stably located on the chromosomes or not. Furthermore, it was also of great interest to determine whether all *C. perfringens* strains contained the full complement of toxin genes, including those for the lethal typing toxins, as it was conceivable that differences in gene expression could account for the occurrence of different biotypes.

I-*Ceu*I, which cleaves each of the ten *rrl* genes of *C. perfringens* CPN50 once (Fig. 1), and does not cut plasmid DNA, is a useful tool for investigating genomic diversity and rapidly locating virulence genes. The results of an I-*Ceu*I analysis performed with 16 isolates of *C. perfringens* are reported, and the localisation of further extrachromosomal virulence genes is described.

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**Table 1** Biotypes of *Clostridium perfringens* and toxins produced

| Toxins | Activities | Gene | Biotype | | | | |
|---|---|---|---|---|---|---|---|
| | | | A | B | C | D | E |
| Major lethal | | | | | | | |
| α | Phospholipase C (lecithinase) | *plc* | + + + | + | + | + | + |
| β | Necrotizing | *cpb* | − | + | + | − | − |
| ε | Permease | *etx* | − | + | − | + | − |
| ι | ADP-ribosylating | *itxAB* | − | − | − | − | + |
| Minor | | | | | | | |
| δ | Hemolysin | ? | − | + | + | − | − |
| θ | $O_2$-labile hemolysin, cytolysin | *pfoA* | + | + | + | + | + |
| κ | Collagenase, gelatinase | *colA* | + | + | + | + | + |
| λ | Protease | *lam* | − | + | − | + | − |
| μ | Hyaluronidase | *nagH* | + | + | + | + | − |
| υ | DNase | ? | + | + | + | + | + |
| Nm | Neuraminidase or sialidase | *nanH, I* | + | + | + | + | + |
| Other | | | | | | | |
| CPE | Enterotoxin, cytotoxin | *cpe* | + | + | + | + | + |

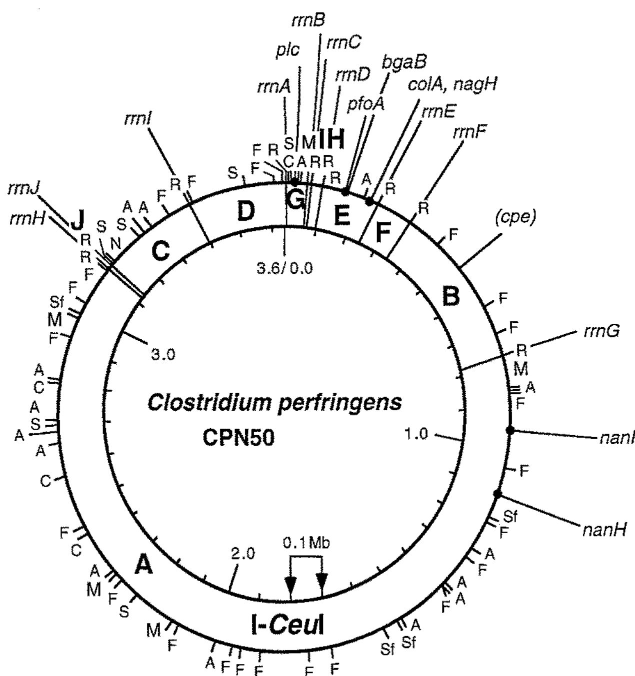


**Fig. 1** A physical map of the *C. perfringens* CPN50 genome including I-*Ceu*I fragments. Restriction enzyme sites; A, *Apa*I; C, *Sac*II (*Ksp*I); F, *Fsp*I (*Avi*II); M, *Mlu*I; N, *Nru*I; R, a 2*Sac*II-*Sma*I-*Nru*I-2*Sma*I-*Nru*I cluster; S, *Sma*I; Sf, *Sfi*I. The positions of virulence genes, *rrn* operons and other key genetic markers are shown

## Materials and methods

### Bacterial strains

All *C. perfringens* strains examined in this study are listed in Table 2.

### Genomic DNA preparation, restriction enzyme digests and pulsed field gel electrophoresis

All genomic DNAs were prepared and digested with I-*Ceu*I as described previously (Canard and Cole 1989; Katayama et al. 1995). I-*Ceu*I was purchased from New England Biolabs and used as recommended. Large DNA fragments were separated by field inversion gel electrophoresis (FIGE) or contour-clamped homogenous electric field electrophoresis (CHEF) as described previously (Canard and Cole 1989). Gels were calibrated with *Saccharomyces cerevisiae* chromosomes (size range 90 to 1600 kb) and a mixture of λ concatemers and *Hin*dIII fragments (New England Biolabs).

### Southern blot hybridization

After electrophoresis gels were processed, DNA transferred to Hybond C-extra filters (Amersham) and hybridized with $^{32}$P-labelled probes as described (Canard and Cole 1989; Canard et al. 1992; Katayama et al. 1995). When a 5′ end-labeled oligomer was used as a probe, the hybridization was carried out at 50° C overnight in 6 × SSC, 1 × Denhardt's solution (0.02% polyvinylpyrrolidone, 0.02% Ficoll 400, and 0.02% bovine serum albumin), 20 mM sodium phosphate buffer (pH 6.8), 0.1% sodium dodecyl sulfate (SDS) containing 200 μg/ml denatured herring sperm DNA (Boehringer Mannheim). All filters were washed twice for 10 min at room temperature in 2 × SSC (1 × SSC is 0.3 M NaCl plus 0.03 M sodium citrate), 0.1% SDS, and twice in 0.2 × SSC, 0.1% SDS.

### Probes

All probe DNAs used in this study were from the laboratory collection or provided by the individuals acknowledged below. The oligonucleotide probe for *itxA* was labeled with [γ-$^{32}$P]ATP and $T_4$ polynucleotide kinase (New England Biolabs), while all other probes were labeled with [α-$^{32}$P]dCTP by using a Nick Translation or Megaprime DNA labelling kit (Amersham).

## Results and discussion

In a recent study (Katayama et al. 1995), the intron-encoded endonuclease, I-CeuI (Marshall and Lemieux 1991) from *C. eugametos*, was shown to cut the 3.6 Mb chromosome of *C. perfringens* CPN50 into 10 fragments that could be resolved by pulsed-field gel electrophoresis (PFGE). These fragments ranged in size

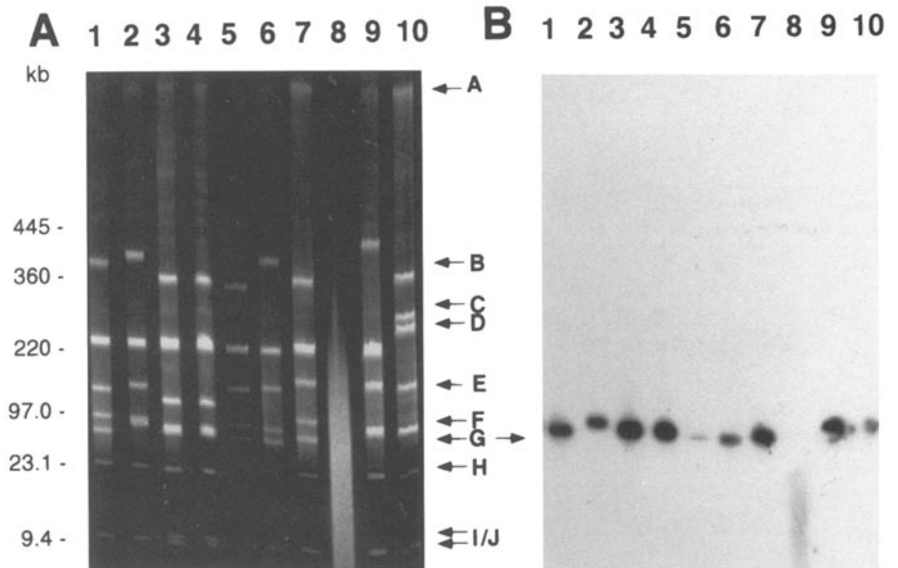


**Fig. 2A, B** Pulsed-field gel electrophoresis of I-*Ceu*I fragments from *C. perfringens* strains. **A** I-*Ceu*I fragments from ten *C. perfringens* strains. Lane 1, CPN50; lane 2, NCTC6785; lane 3, NCTC8798; lane 4, 8–6; lane 5, A99; lane 6, G23; lane 7, CN3922; lane 8, CWC235; lane 9, NCTC2062; lane 10, ATCC27324. **B** The gel in **A** was blotted and hybridized with a *plc* gene probe. Note extensive degradation of DNA from CWC235 (lane 8) due to production of stable nuclease (Canard et al. 1992)

from >2 Mb (A) to 9.5 kb (I/J) and all ten I-*Ceu*I cleavage sites were located in *rrl* genes (Katayama et al. 1995). The I-*Ceu*I restriction map of this strain, based on the present physical map (Katayama et al. 1995), is shown in Fig. 1. As all of the I-*Ceu*I cleavages occurred specifically in the *rrl* genes this enzyme could be a very powerful tool for investigating genomic diversity among *C. perfringens* strains. The digestion patterns of the genomes of 16 clinical isolates, nine of which were CPE producers, were compared (Figs. 2A, 3A). All strains yielded a conserved pattern of 10 I-*Ceu*I fragments A to J (Figs. 2A, 3A) although some fragment length variation was seen.

## Rapid mapping of virulence genes located on the chromosome

All of the I-*Ceu*I fragments, with the exception of fragment A, are suitable in size for rapidly mapping genes on the chromosome of *C. perfringens*. To date, five virulence genes (*colA*, *cpe*, *nagH*, *pfoA*, and *plc*) have been mapped in *C. perfringens* CPN50 (Fig. 1) or 8-6 (in the case of *cpe*) (Canard and Cole 1989; Canard et al. 1992; Katayama et al. 1995). Their chromosomal positions were confirmed here by means of I-*Ceu*I mapping and hybridization and the analysis was extended to include all 16 isolates. Typical results obtained with

**Table 2** *C. perfringens* strains used

| Strain | Biotype | Origin | Year of isolation | Enterotoxin | Location of *cpe*[a] | IS*1151* |
|---|---|---|---|---|---|---|
| CPN50 | A | Humans, USA | 1940s | – | | |
| NCTC6785 | A | Humans, USA | 1944 | – | | |
| A99 | A | Humans, Germany | 1986 | – | | |
| NCTC8798 | A | Humans, England | 1953 | + | Chromosome | – |
| 8-6 | A | Spontaneous mutant of NCTC8798 | 1972 | + | Chromosome | – |
| NCTC9851 | A | Humans, England | 1956 | + | Chromosome | – |
| NCTC10239 | A | Humans, England | 1961 | + | Chromosome | – |
| CP1088 | A | Humans, France | 1992 | + | Chromosome | – |
| CP192-81 | A | Humans, France | 1981 | + | Chromosome | – |
| F3686/80 | A | Humans, England | 1980 | + | Plasmid | + |
| 44071-CO5 | A | Goats, Belgium | 1991 | + | Plasmid | + |
| 88L662MF | A | Pigs, Belgium | 1988 | + | Plasmid | + |
| 91E01793F | A | Cattle, Belgium | 1991 | + | Plasmid | + |
| G23 | B | Calves, France | 1950s | – | | – |
| CN3922 | B | Pigs, Belgium | 1989 | – | | + |
| CWC235 | C | Pigs, Denmark | ? | – | | + |
| 945P | D | Sheep, Belgium | 1989 | + | Plasmid | + |
| NCTC2062 | D | Sheep, England | ?[b] | – | | + |
| ATCC27324 | E | Rabbits, USA | 1960s | – | | + ? |

[a] Cornillot et al. (1995)
[b] Uncertain

**Table 3** The locations of virulence genes in *C. perfringens* strains

| Strain | Biotype | I-*Ceu*I fragments hybridized with toxin gene probes | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | α *plc* | β *cbp* | ε[a] *etx* | ι *itx* | θ *pfoA* | κ *colA* | λ *lam* | μ *nagH* | CPE *cpe* |
| CPN50 | A | G | | – | – | E | E | – | E | – |
| NCTC6785 | A | G | | – | – | E | E | – | E | – |
| A99 | A | G | | – | – | E | E | – | E | – |
| G23 | B | G | ? | – | – | E | E | – | E | – |
| CN3922 | B | G | ? | P | – | E | E | – | E | – |
| CWC235 | C | ND | P | – | – | ND | ND | – | ND | – |
| NCTC2062 | D | G | | P | – | E | E | P | E | – |
| ATCC27324 | E | G | | – | P | E | E | – | E | – |
| NCTC8798 | A | G | | – | – | – | E | – | – | B |
| 8-6 | A | G | | – | – | – | E | – | – | B |
| NCTC9851 | A | G | | – | – | – | E | – | – | B |
| NCTC10239 | A | G | | – | – | – | E | – | – | B |
| 945P | D | G | | P | – | E | E | P | E | P |
| F3686/80 | A | G | | – | – | E | E | – | E | P |
| 44071-CO5 | A | G | | – | – | E | E | – | E | P |
| 88L662MF | A | G | | – | – | E | E | – | E | P |
| 91E01793F | A | G | | – | – | E | E | – | E | P |

[a] Canard et al. (1992); Cornillot et al. (1995)
P, plasmid; ND, Could not be determined due to nuclease production

*plc*- and *nagH*-specific probes are presented in Figs. 2B, and 3B and the findings for the main virulence genes are summarized in Table 3. The positions of four virulence genes (*colA*, *nagH*, *pfoA*, and *plc*) are highly conserved, despite their being located on I-*Ceu*I fragments of variable length (fragments B, E, or G; Fig. 2A), as reported previously (Canard et al. 1992; Katayama et al. 1995).

## Studies on enterotoxin-producing strains

The enterotoxin gene, *cpe*, may be localized either on large plasmids or on the chromosome (Cornillot et al. 1995). This is illustrated in Fig. 3B, where it can be seen that in four strains *cpe* was found on I-*Ceu*I fragment B, which varied in size, while the more complex hybridization pattern characteristic of plasmids, which are unaffected by I-*Ceu*I digestion, was seen with five other strains. Interestingly, on examination of the four CPE-producing strains which have *cpe* genes on chromosomal fragment B, it was found that two separate deletions had occurred in fragment E, which was ~20 kb smaller than that of the other strains, and in all cases both the *nagH* (Fig. 3C) and *pfoA* genes (data not shown) had been deleted (Table 3). However, the *bgaB* gene, encoding β-galactosidase, which is located 5.4 kb downstream from *pfoA* (Katayama et al. 1995), the collagenase gene, *colA*, which is normally situated near *nagH*, and the sequences flanking *nagH* were still present (see Fig. 1). The size of the *nagH* deletion was estimated as 1.9–10.0 kb (data not shown). By contrast, both *nagH* (Fig. 3C) and *pfoA* (data not shown) were present in the strains harboring *cpe* on a plasmid (Table 3). Enterotoxin producers with chromosomal or plasmid-borne *cpe* genes can also be distinguished on the basis of their I-*Ceu*I profiles, as the I/J doublet was clearly separated in the case of the former group (Fig. 3A) whereas in all other *C. perfringens* strains it was not resolved under these conditions (Figs. 2A, 3A).

To establish whether this was a general trend in *C. perfringens* strains with chromosomal *cpe* genes or possibly due to the fact that these food-poisoning strains, which were all isolated in England in the '50s and '60s, may have been related, we also examined two food-poisoning strains of recent French origin, CP1088 and CP192-81 (Table 2). Again, *cpe* was found to be located on I-*Ceu*I fragment B but neither *pfoA* nor *nagH* could be detected (data not shown).

The marked dichotomy between *C. perfringens* strains with chromosomal or plasmid-borne *cpe* genes is intriguing and may be of relevance to *C. perfringens* enterotoxemia. It was shown very recently that strains with chromosomal *cpe* genes are associated with human food poisoning, whereas those with episomal enterotoxin genes were involved in veterinary pathologies (Cornillot et al. 1995).

## Localization of ε-, β-, ι-, and λ-toxin genes on plasmids

Circular plasmids migrate abnormally in pulsed-field gels (Smith et al. 1986) and exhibit characteristic profiles (Cornillot et al. 1995) that are not altered by I-*Ceu*I digestion (Fig. 3B). This facilitates the localization of certain virulence genes, and in earlier work (Canard et al. 1992; Cornillot et al. 1995) the *etx* gene encoding the λ-toxin was shown to be plasmid-borne in strains 945P and NCTC2062 (Table 1).

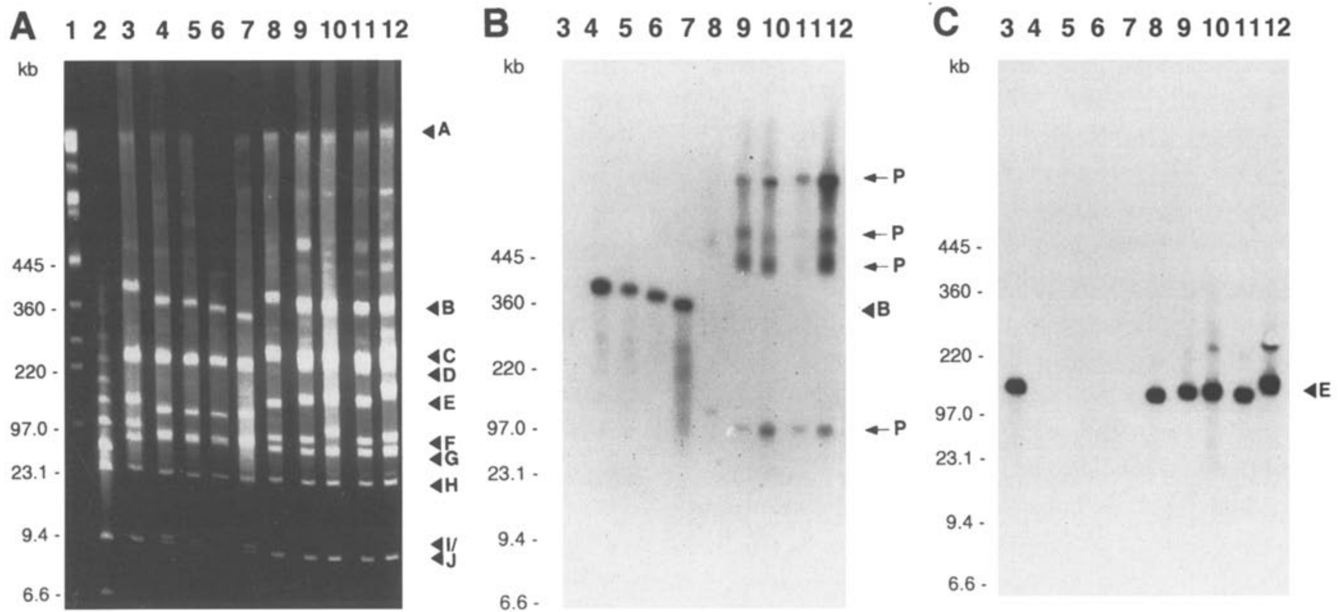


**Fig. 3A–C** Pulsed-field gel electrophoresis of I-*Ceu*I fragments from CPE-producing *C. perfringens* strains. **A** I-*Ceu*I fragments from CPN50 and nine CPE-producing *C. perfringens* strains. Lane 1, *S. cerevisiae* chromosomes; lane 2, $\lambda$ concatemers and *Hin*dIII markers; lane 3, CPN50 (CPE−); lane 4, NCTC8798; lane 5, 8–6; lane 6, NCTC9851; lane 7, NCTC10239; lane 8, 945P; lane 9, F3686/80, lane 10, 44071-CO5; lane 11, 88L662MF; lane 12, 91E01793F. **B** Southern blot of the gel shown in **A** hybridized with a *cpe* gene probe. Note presence of *cpe* on chromosomal fragment B (lanes 4–7) and on plasmids (P) (Cornillot et al. 1995), giving multiple bands (lanes 8–12). **C** Same gel hybridized with *nagH* gene probe

The genes for the $\beta$-, $\iota$-, and $\lambda$-toxins (*cpb*, *itxAB*, and *lam*) have been cloned and sequenced recently (Hunter et al. 1993; Jin et al. 1996; Perelle et al. 1993) but their locations (chromosome or plasmid) have not yet been established. Consequently, localization experiments were performed using both intact genomic DNA and I-*Ceu*I fragments and typical results with the type E strain ATCC27324 (Table 1) are shown in Fig. 4. Bands corresponding to two plasmids of ~95 and 130 kb were apparent in both undigested (lane 3) and I-*Ceu*I-treated samples (lane 4) and the 130-kb species hybridized with the *itxA* probe. Similar results were obtained with a *lam* probe and indicated that the gene was located on plasmids of 140 kb and 120 kb in two type D strains, NCTC2062 and 945P (Table 3), respectively.

No hybridization signals were detected when the *cpb* probe was used with DNA from two type B isolates, suggesting that this gene may have been lost. Consequently, the type C strain, CWC235, a known $\beta$-toxin producer, was examined and the gene was detected on a plasmid (Fig. 5) where it was linked to a copy of the transposable element IS*1151* (Daube et al. 1993). All attempts to analyse this strain by PFGE have been unsuccessful (Canard et al. 1992).

## Genetic basis for biotyping

The major conclusion of the current mapping analysis concerns the localization of the genes for a number of toxins and related virulence factors. It is now clear that there is extensive diversity in the toxin gene arsenal and that differences in *C. perfringens* biotype reflect the presence or absence of the genes for three of the four lethal typing toxins, as has also been demonstrated by PCR typing (Daube et al. 1994), rather than differences in gene expression.

The conserved chromosomal location of the $\alpha$-toxin gene, *plc*, near the origin of replication (Canard and Cole 1989; Canard et al. 1992) and the localization of the genes for the $\beta$, $\varepsilon$, and $\iota$-toxins on plasmids provides a logical basis for explaining the biotype drift that is sometimes observed among *C. perfringens* strains (McDonel 1986). The unequal distribution of these plasmids, and their ability to be lost or acquired, would account for changes in biotype. The combined results of this work show that at least five virulence genes are plasmid-borne (Table 3) and the variation seen in the sizes of the various plasmids raises the possibility that they may carry additional genes involved in pathogenesis, such as that for $\delta$-toxin (Table 1), or possibly in host range. It is also striking that IS*1151* is linked to the plasmid-borne *cpe* and *etx* genes (Cornillot et al. 1995), to *cpb* in strain CWC235 (Fig. 5) and the *itxAB* operon (data not shown).

## Possible relevance to other toxigenic clostridia

The association of toxin genes with extrachromosomal elements in some pathogenic clostridia is well

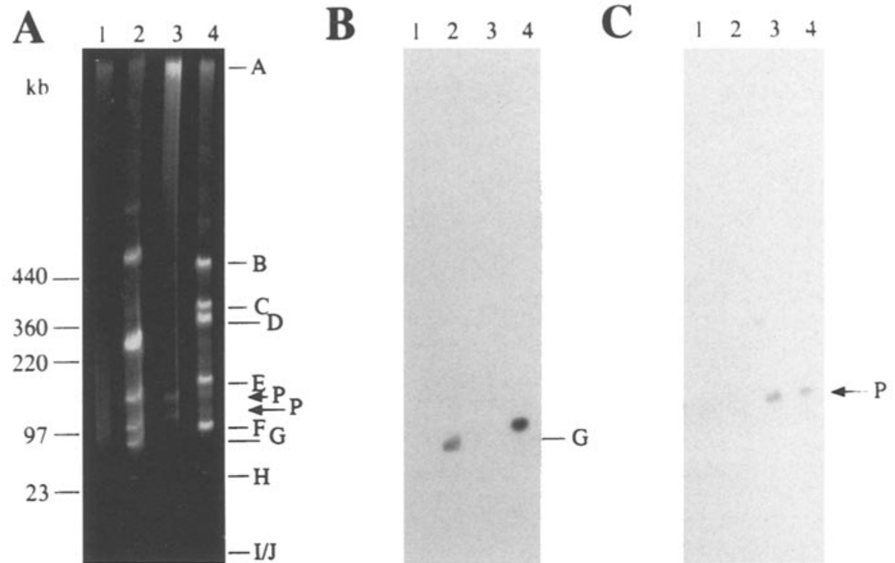


**Fig. 4A–C** Localization of ι-toxin (*itxAB*) gene. **A** Pulsed-field gel electrophoresis of I-*Ceu*I fragments (A–J) from *C. perfringens* type A and type E strains. Lane 1, undigested CPN50 DNA ($\iota^-$); lane 2, CPN50 ($\iota^-$) I-*Ceu*I digest; lane 3, undigested DNA from type E strain ATCC27324 ($\iota^+$); lane 4, ATCC27324 ($\iota^+$) I-*Ceu*I digest. **B** Southern blot of the gel shown in **A**, hybridized with a *plc* gene probe. **C** Same blot rehybridized with a *itxA* gene probe. Positions of plasmids (P) are indicated

documented (Eklund 1992). The genes for the botulinum neurotoxin are associated with phages in *C. botulinum* types C and D, and plasmids in type G strains. There is also compelling evidence for the idea that production of α-toxin in *C. novyi* results from lysogenisation with phages (Eklund 1992), while the gene for the tetanus toxin is known to reside on a plasmid in *C. tetani* (Finn et al. 1984). Our findings that the genes for the β, ε, and ι-toxins, as well as those for λ-toxin and the enterotoxin, are situated on episomes indicates that the contribution of extrachromosomal genetic material to the pathogenicity of Clostridia is much greater than was initially realised. At present it is unclear whether these genes are located on bona fide plasmids or on phages that exist extrachromosomally. The marked instability (Duncan et al. 1978; M. Popoff, personal communication) seen with the β-toxin gene is reminiscent of a relationship such as the pseudo-lysogeny which is often observed with certain neurotoxin-producing *C. botulinum* strains (Eklund 1992). It will be of great interest to determine whether some, or all, of the toxin genes present in other pathogenic clostridial species, such as *C. difficile*, are also located extrachromosomally.

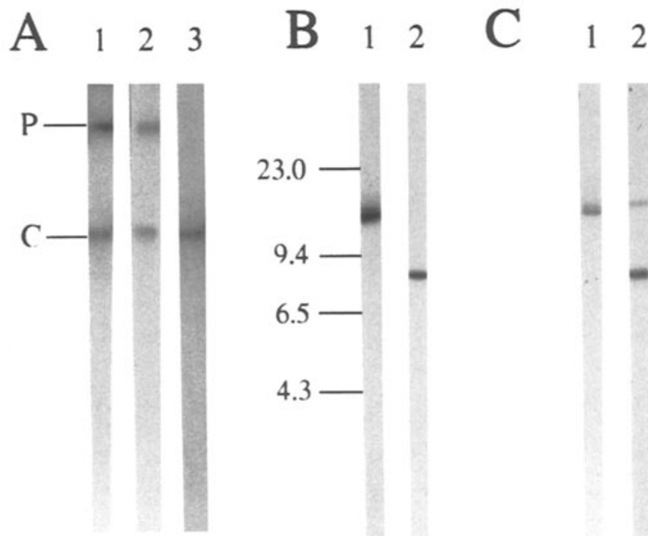


**Fig. 5A–C** Localization of the *cpb* gene on a plasmid in strain CWC235. **A** Plasmid DNA was extracted as described (Cornillot et al. 1995) and hybridized with probes for *cpb* (lane 1), IS*1151* (lane 2) and *plc* (lane 3). The positions of chromosomal (C) and plasmid (P) DNAs are indicated. Chromosomal DNA bands often contain sheared plasmid DNA and may thus give additional hybridization signals. **B** Plasmid DNA was digested with *Eco*RI (lane 1), and *Eco*RV (lane 2), and subjected to Southern hybridization using a probe for *cpb*. **C** Same blot hybridized with an IS*1151*-specific probe. Note the linkage between these markers

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