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Inactivation of the gene (*cpe*) encoding *Clostridium perfringens* enterotoxin eliminates the ability of two *cpe*-positive *C. perfringens* type A human gastrointestinal disease isolates to affect rabbit ileal loops

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Summary

Previous epidemiological studies have implicated *Clostridium perfringens* enterotoxin (CPE) as a virulence factor in the pathogenesis of several gastrointestinal (GI) illnesses caused by *C. perfringens* type A isolates, including *C. perfringens* type A food poisoning and non-food-borne GI illnesses, such as antibiotic-associated diarrhoea and sporadic diarrhoea. To further evaluate the importance of CPE in the pathogenesis of these GI diseases, allelic exchange was used to construct *cpe* knock-out mutants in both SM101 (a derivative of a *C. perfringens* type A food poisoning isolate carrying a chromosomal *cpe* gene) and F4969 (a *C. perfringens* type A non-food-borne GI disease isolate carrying a plasmid-borne *cpe* gene). Western blot analyses confirmed that neither *cpe* knock-out mutant could express CPE during either sporulation or vegetative growth, and that this lack of CPE expression could be complemented by transforming these mutants with a recombinant plasmid carrying the wild-type *cpe* gene. When the virulence of the wild-type, mutant and complementing strains were compared in a rabbit ileal loop model, sporulating (but not vegetative) culture lysates of the wild-type isolates induced significant ileal loop fluid accumulation and intestinal histopathological damage, but neither sporulating nor vegetative culture lysates of the *cpe* knock-out mutants induced these intestinal effects. However, full sporulation-associated virulence could be restored by complementing these *cpe* knock-out mutants with a recombinant plasmid carrying the wild-type *cpe* gene, which confirms that the observed loss of virulence for the *cpe* knock-out mutants results from the specific inactivation of the *cpe* gene and the resultant loss of CPE expression. Therefore, *in vivo* analysis of our isogenic *cpe* mutants indicates that CPE expression is necessary for these two *cpe*-positive *C. perfringens* type A human disease isolates to cause GI effects in the culture lysate:ileal loop model system, a finding that supports CPE as an important virulence factor in GI diseases involving *cpe*-positive *C. perfringens* type A isolates.

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Introduction

The virulence of *Clostridium perfringens*, a Gram-positive, anaerobic, spore-forming bacterium, derives in large part from its notorious toxin-producing abilities. At least 14 different *C. perfringens* toxins have been reported in the literature (McDonel, 1986; Hatheway, 1990; Gibert *et al*., 1997; Rood, 1998); however, each individual *C. perfringens* isolate produces only a subset of these 14 toxins. This variability in toxin gene expression forms the basis for a scheme that classifies *C. perfringens* isolates into one of five types (A–E), based upon an isolate's ability to produce alpha, beta, epsilon and iota toxins (McDonel, 1986; Hatheway, 1990). Type A isolates of *C. perfringens*, which produce alpha but not beta, epsilon or iota toxin, are important causes of both gastrointestinal (GI) and histotoxic infections in humans and domestic animals (Hatheway, 1990; Songer, 1996).

The gene (*cpe*) encoding another of the 14 *C. perfringens* toxins, i.e. *C. perfringens* enterotoxin (CPE), is present in less than 5% of global *C. perfringens* isolates (Kokai-Kun *et al*., 1994), most of which classify as type A isolates (Songer and Meer, 1996). Although relatively rare, these *cpe*-positive *C. perfringens* type A isolates hold considerable pathogenic importance because of their established role as major causes of GI illness in both humans and domestic animals. For over 30 years, these bacteria have been implicated as the causative agents of

C. perfringens type A food poisoning (McClane, 1997), which annually ranks as the second most commonly reported food-borne disease in developed countries (Bean *et al*., 1996). More recently, *cpe*-positive *C. perfringens* type A isolates have also become recognized as a major cause of non-food-borne human GI diseases, including antibiotic-associated diarrhoea and sporadic diarrhoea (Carman, 1997).

Epidemiological evidence suggests that CPE plays an important role in the pathogenesis of both the food-borne and non-food-borne human GI illnesses caused by *cpe*-positive *C. perfringens* type A isolates. For example, previous studies (Batholomew *et al*., 1985; Birkhead *et al*., 1988) have demonstrated that a high percentage of diarrhoeal faeces from patients suffering from food-borne and non-food-borne GI illnesses involving *cpe*-positive *C. perfringens* type A isolates contain CPE, often at concentrations resembling those known to cause GI effects in animal models. Furthermore, the ingestion of highly purified CPE by human volunteers has been shown to induce diarrhoea and abdominal cramps (Skjelkvale and Uemura, 1977), which are the predominant symptoms of the human GI illnesses involving *cpe*-positive *C. perfringens* type A isolates.

While such epidemiological correlations are informative, they are insufficient to establish formally that CPE is a necessary contributor to the GI symptoms of either *C. perfringens* type A food poisoning or the non-food-borne human GI diseases associated with *cpe*-positive *C. perfringens* type A isolates. CPE could be only one of several toxins contributing to the pathogenesis of these human GI illnesses, particularly given (i) the large number of *C. perfringens* toxins already identified; (ii) the recent discovery of a new *C. perfringens* toxin (Gibert *et al*., 1997) named beta-2, which brings into question whether the entire repertoire of *C. perfringens* toxins has yet been established; (iii) that at least some variants of *C. perfringens* alpha toxin, which is expressed by *C. perfringens* type A isolates, have been implicated (Ginter *et al*., 1996) as important virulence factors in enteric disease of domestic animals; and (iv) epidemiological reports (Carman, 1997) indicate that the symptoms of non-food-borne human GI diseases associated with *cpe*-positive *C. perfringens* type A isolates are typically more severe and longer lasting than those of *C. perfringens* type A food poisoning, which could suggest that additional toxins besides CPE might contribute, in particular, to the pathogenesis of non-food-borne human GI diseases involving *cpe*-positive *C. perfringens* type A isolates. That possibility receives further indirect support from recent studies (Collie and McClane, 1998) indicating that genotypic differences exist between *cpe*-positive *C. perfringens* type A isolates responsible for non-food-borne disease and those responsible for food poisoning.

In order to evaluate the contribution of CPE to the GI pathogenesis of *cpe*-positive *C. perfringens* type A isolates better, our current study prepared an isogenic *cpe* knock-out mutant of SM101, which is an electroporatable derivative of the *C. perfringens* type A food poisoning isolate NCTC 8798 that carries a chromosomal *cpe* gene, as well as F4969, which is a *C. perfringens* type A non-food-borne disease isolate that carries a plasmid-borne *cpe* gene. Lysates from both vegetative and sporulating cultures of these two *cpe* knock-out mutants, as well as the parent strains and complementing strains of each mutant transformed with a low-copy-number plasmid carrying the cloned *cpe* gene (Czeczulin *et al*., 1996), were tested for their ability to cause fluid accumulation and histological damage in rabbit ileal loops. The results from these experiments now provide clear and direct evidence that, at least in the concentrated lysate:rabbit ileal loop model system used for these studies, CPE expression is necessary for both SM101 and F4969 to cause both fluid accumulation and histopathological damage. By extension, these results are consistent with CPE playing an important role in the pathogenesis of human GI diseases induced by SM101 and F4969 (and presumably by other *cpe*-positive *C. perfringens* type A isolates) and also fail to implicate other toxins in the GI effects caused by SM101 or F4969.

Results

Construction of cpe *knock-out mutants*

To create pMRS103 (see Fig. 1), the ~0.4 kb *Bgl*II–*Nhe*I internal *cpe* gene fragment present in pMRS102 was removed and replaced with the ~1.3 kb *Sma*I–*Nae*I fragment of pJRC100 (see Table 3), which contains a chloramphenicol resistance determinant (*catP*). This plasmid was transformed into *C. perfringens* strain SM101 (an electroporatable derivative of food poisoning isolate NCTC 8798, which carries a chromosomal *cpe* gene; data not shown) or F4969 (a non-food-borne disease isolate, which carries a plasmid-borne *cpe* gene; Collie *et al*., 1998), and transformants were selected on brain–heart infusion (BHI) agar plates containing chloramphenicol and erythromycin. As pMRS103 does not have a replication origin for *C. perfringens*, these transformants should have integrated the chloramphenicol resistance determinant into their *cpe* gene by homologous recombination (Fig. 1). A double cross-over event between this mutated *cpe* gene and the wild-type *cpe* gene was then obtained by selecting chloramphenicol-resistant, but erythromycin-sensitive, clones from these chloramphenicol- and erythromycin-resistant transformants. The putative mutant derivatives of SM101 and F4969 were designated MRS101 and MRS4969 respectively.

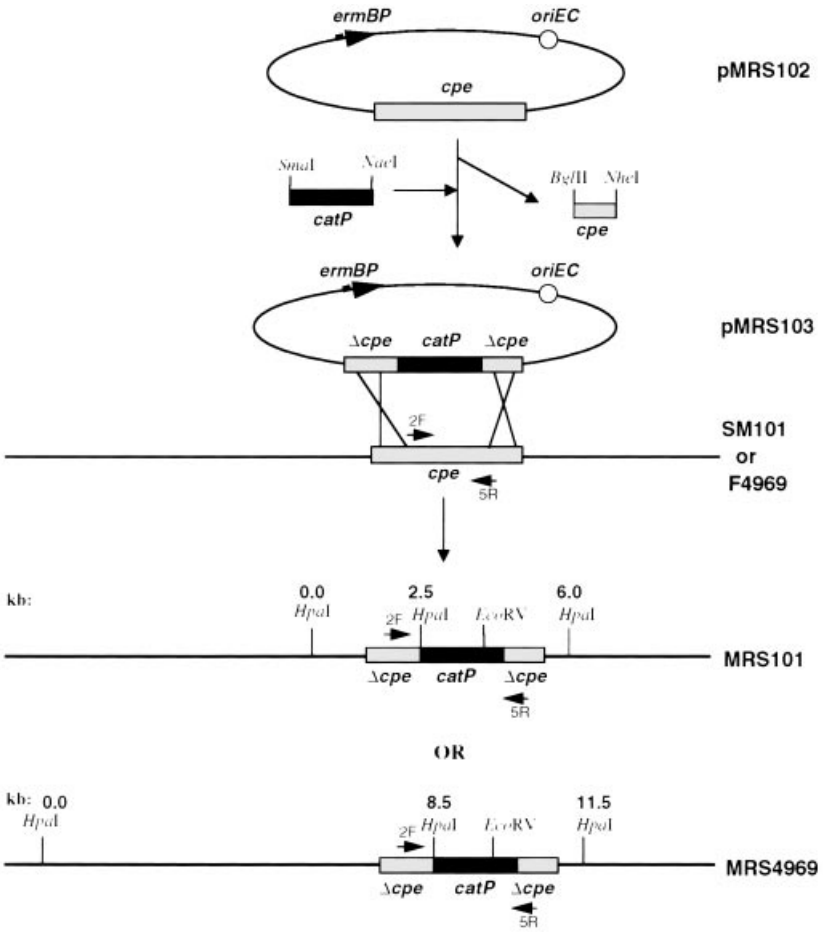

Fig. 1. Construction of pMRS103 and inactivation of the *cpe* gene in *C. perfringens*. The internal 0.4 kb *Bgl*II–*Nhe*I fragment of the wild-type *cpe* gene was removed from pMRS102 and replaced by a 1.3 kb *Sma*I–*Nae*I fragment, which carries a chloramphenicol resistance determinant (*catP*) from pJRC100 (Table 3). The resulting plasmid, pMRS103, was then transformed into *C. perfringens* strains SM101 or F4969 by electroporation, and *cpe* mutants of both strains were isolated by allelic exchange. The diagram indicates the process by which the wild-type *cpe* gene from SM101 or F4969 was replaced by the Δ*cpe*::*catP* region from pMRS103, resulting in the independent construction of the *cpe* mutants MRS101 or MRS4969 respectively. 2F and 5R indicates the location of *cpe* internal primers used for PCR analysis.

PCR analysis of cpe *knock-out mutants*

The putative identities of MRS101 and MRS4969 as *cpe* knock-out mutants were first evaluated by polymerase chain reaction (PCR) analysis of DNA isolated from these mutants (data not shown). Using a standard *cpe* PCR detection protocol (Kokai-Kun *et al*., 1994), an ~0.6 kb *cpe* PCR product was amplified using template DNA isolated from either wild-type strain SM101 or F4969. In contrast, an ~1.5 kb PCR product was obtained when the same PCR amplification reaction was performed using DNA isolated from either mutant MRS101 or MRS4969. These PCR results are consistent with the ~0.4 kb internal *cpe* fragment of mutant strains MRS101 and MRS4969 having been replaced with the 1.3 kb *catP*-containing fragment of the mutator plasmid pMRS103. This conclusion receives further support from the observed amplification of a similar 1.5 kb PCR product using pMRS103 as template DNA.

Southern blot analysis of cpe *knock-out mutants*

To confirm the genetic identity of the putative *cpe* mutant strains MRS101 and MRS4969 further, total purified DNA from wild-type and mutant strains was digested with *Hpa*I. Three identical Southern blots were prepared using this digested DNA, and these blots were hybridized, separately, with probes specific for the *cpe*, *catP* genes or pJIR418 sequences (Fig. 2).

These analyses showed that an ~5 kb *Hpa*I fragment from wild-type strain SM101 hybridized with our *cpe*-specific probe; however, no hybridizing fragment of this size was found in the mutant strain MRS101. Instead, two hybridizing bands (of ~3.5 kb and 2.5 kb) were observed with DNA from this strain (Fig. 2A). This profile is consistent with results expected if MRS101 had replaced the ~0.4 kb internal *cpe* sequences of wild-type strain SM101 with the ~1.3 kb *catP*-containing fragment of pMRS103, which has an internal *Hpa*I restriction site that should produce fragments of 3.5 kb and 2.5 kb after *Hpa*I digestion. The 3.5 kb *Hpa*I fragment from MRS101 also hybridized with our *catP*-specific probe (Fig. 2A), but this same *catP*-specific probe did not hybridize with DNA from wild-type strain SM101. The final evidence that only the inactivated *cpe* gene of pMRS103 remains integrated into the chromosome of MRS101, and thus a double cross-over event had occurred (Fig. 1), was obtained from the observation that

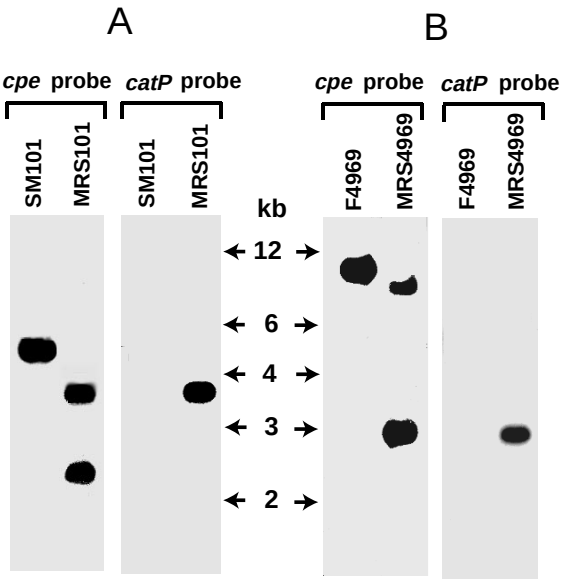

Fig. 2. Southern hybridization analysis of total DNAs from *C. perfringens* wild-type and *cpe* mutant strains. DNA prepared from wild-type (SM101 and F4969) and *cpe* mutant (MRS101 and MRS4969) *C. perfringens* strains was digested with *Hpa*I, subjected to electrophoresis, blotted and probed separately with either *cpe*-specific or *catP* -specific DIG-labelled probes.
A. DNAs from wild-type SM101 and its *cpe* mutant strain MRS101.
B. DNAs from F4969 and its *cpe* mutant strain MRS4969.
Molecular sizes of the DNA markers are given in the middle.

DNA from the MRS101 mutant did not hybridize with a probe consisting of labelled vector pJIR418 DNA sequences (data not shown).

When similar Southern hybridization experiments were performed on total DNA of wild-type F4969 and mutant MRS4969, an ~10.5 kb *Hpa*I fragment from wild-type strain F4969 was found to hybridize with our *cpe*-specific probe (Fig. 2B). This difference, i.e. localization of the *cpe* gene on a 10.5 kb *Hpa*I fragment of F4969 DNA instead of the 5 kb *Hpa*I fragment observed with SM101 DNA, is consistent with previous observations (Collie and McClane, 1998), indicating that the *cpe* restriction fragment length polymorphism (RFLP) pattern of non-food-borne disease isolate F4969 differs from that of food-poisoning isolates such as NCTC 8798, the parent strain of SM101. Our *cpe* probe did not hybridize with a 10.5 kb fragment in the mutant MRS4969 digest but, instead, hybridized to two bands of ~8.5 kb and 3 kb (Fig. 2B). Our *catP*-specific probe hybridized with an ~3 kb fragment of *Hpa*I-digested MRS4969 DNA but, as expected, no hybridizing band was observed with DNA from the F4969 wild-type strain. A final piece of evidence supporting the predicted double cross-over event between the wild-type *cpe* gene and the Δ*cpe*::*catP* segment from pMRS103 (Fig. 1) is the observation that a pJIR418-specific probe did not hybridize with MRS4969 DNA (data not shown).

Evaluation of CPE expression by cpe *knock-out mutants*

Having obtained evidence for successful construction of two *cpe* knock-out mutants, we next confirmed that both MRS101 and MRS4969 are unable to express CPE. When control experiments were performed to confirm the specificity and reliability of the CPE Western immunoblot assay for assessing CPE expression by our mutants, no detectable CPE expression was observed in vegetative cultures of wild-type strains SM101 or F4969 (data not shown). However, a 35 kDa immunoreactive band co-migrating with purified CPE was detected in lysates prepared from sporulating cultures of SM101 and F4969 (Fig. 3). Furthermore, densitometric analyses of these Western blots revealed that, under the growth conditions used in this experiment, CPE expression was ~fivefold higher in F4969 compared with SM101 (Table 1). Collectively, these results are consistent with previous studies (Czeczulin *et al*., 1993; 1996; Kokai-Kun *et al*., 1994; Collie

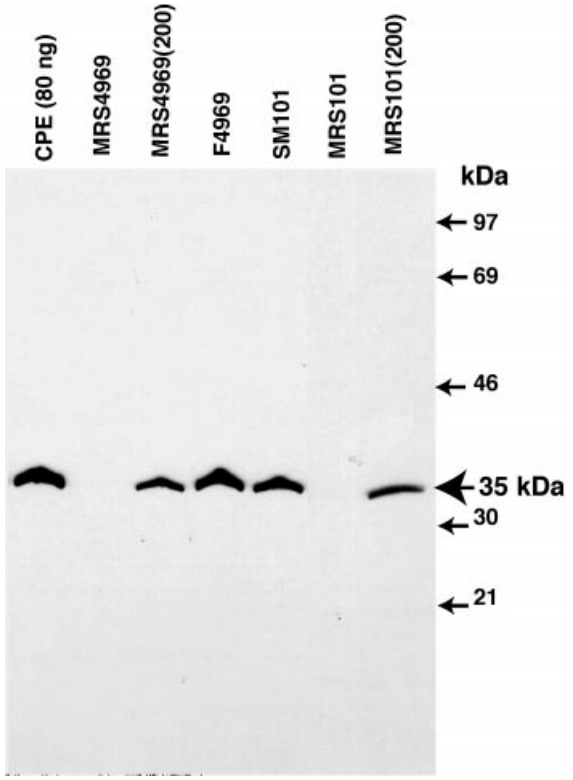

Fig. 3. Western immunoblot analysis of sporulating *C. perfringens* lysates. Lysates were analysed for CPE expression by immunoblot analysis using CPE antiserum. Each lane contains lysate from sporulating DS cultures of: wild-type strains SM101 (5 μl) or F4969 (1 μl), *cpe* mutant strains MRS101 (40 μl) or MRS4969 (40 μl), or the complementing strains MRS101(pJRC200) (5 μl) or MRS4969 (pJRC200) (1 μl). For comparison, 80 ng of purified native CPE is shown in the leftmost lane of the blot. The arrowhead indicates the migration of purified CPE when stained by Coomassie blue (not shown). Molecular sizes of the protein markers are given to the right of the blot.

Table 1. Quantification of CPE expression, growth and sporulation by *C. perfringens* strains grown in Duncan–Strong (DS) sporulation medium.

Culture	CPE expression[a] ($\mu g\,ml^{-1}$)	Total cells ml^{-1}[b] (10^7)	Spores ml^{-1} (10^7)
F4969	47 ± 5	12 ± 2	10 ± 1
MRS4969	0	13 ± 2	10 ± 1
MRS4969(pJRC200)	63 ± 5	16 ± 5	12 ± 2
MRS4969(pJIR418)	0	11 ± 3	10 ± 2
SM101	10 ± 2	8 ± 3	6 ± 1
MRS101	0	10 ± 2	8 ± 3
MRS101(pJRC200)	21 ± 2	9 ± 5	8 ± 5
MRS101(pJIR418)	0	9 ± 2	8 ± 2

a. Results shown are based upon at least three independent determinations for each experimental parameter for each culture.
b. 'Total cells' represents the sum of vegetative cells and sporulating cells present in each culture.

et al., 1998), demonstrating that CPE expression is strongly associated with sporulation and that CPE expression varies considerably among *cpe*-positive wild-type strains.

When the same CPE Western immunoblot procedure was applied to culture lysates of *cpe* mutant strains MRS101 and MRS4969, no CPE-specific immunoreactivity was detected against lysates prepared from vegetative (data not shown) or sporulating cultures (Fig. 3) of MRS101 or MRS4969, even when 50-fold-concentrated lysates of these two mutants were analysed.

As CPE expression is strongly associated with sporulation, we also compared the sporulation capability of each mutant against that of its wild-type parent, in order to ensure that the failure of our knock-out mutants to express CPE could not be ascribed to their inability to sporulate. Using microscopic counting techniques, the numbers of vegetative and sporulating cells present per millilitre of Duncan–Strong (DS, which induces sporulation, results shown in Table 1) or fluid thioglycollate (FTG, which supports vegetative growth; data not shown) cultures were very similar for each knock-out mutant and its wild-type parent, i.e. deletion of the *cpe* gene did not significantly affect either growth or sporulation of our knock-out mutants relative to their wild-type parents. These results confirm that the loss of CPE expression by mutant strains is not the result of a sporulation defect, but is instead caused by inactivation of the *cpe* gene itself.

Complementation of cpe *knock-out mutations*

To ensure that any *in vivo* virulence effects that might be observed for our *cpe* knock-out mutants were not caused by polar mutations, we introduced plasmid pJRC200, which carries the intact wild-type *cpe* gene (Table 3), into MRS101 or MRS4969 cells. After chloramphenicol- and erythromycin-resistant colonies were selected, *cpe* PCR analysis confirmed the presence of pJRC200 in both the MRS101 and the MRS4969 transformants, which were then named MRS101(pJRC200) and MRS4969(pJRC200). Our *cpe* PCR assay yielded (data not shown) two amplified products from each of these pJRC200 transformants. The size of these two PCR products corresponds exactly to the ~0.6 kb wild-type *cpe* product that should be amplified from the wild-type *cpe* gene present in pJRC200, and to the ~1.5 kb product that should be amplified from the mutated *cpe* gene present in the mutant host strains MRS101 and MRS4969 (see Fig. 1). The shuttle vector pJIR418 was also introduced into MRS101 and MRS4969, and the presence of pJIR418 in MRS101(pJIR418) and MRS4969 (pJIR418) was confirmed by Southern analysis using a pJIR418-specific probe (data not shown).

CPE Western immunoblot analyses (Fig. 3) confirmed that plasmid pJRC200 could complement the Δ*cpe*::*catP* mutation in MRS101 and MRS4969. Both the MRS101(pJRC200) and the MRS4969(pJRC200) transformants were able to express CPE in a sporulation-associated manner reminiscent of CPE expression by wild-type strains SM101 and F4969, i.e. neither pJRC200 transformant produced detectable levels of CPE during vegetative growth (data not shown), but did produce CPE when sporulating (Fig. 3). Densitometric analyses of these Western blots revealed that, under the growth conditions used in this experiment, MRS4969(pJRC200) produced about threefold more CPE than MRS101(pJRC200) (Table 1). These results are similar to the patterns obtained with the two *cpe*-positive wild-type parent strains, in which F4969 was shown to produce fivefold more CPE than SM101. CPE Western immunoblots also demonstrated, as expected, that CPE expression (data not shown) was absent from both vegetative and sporulating cultures when the shuttle plasmid pJIR418 alone was introduced into MRS101 or MRS4969, confirming that the observed CPE expression in MRS101(pJRC200) and MRS4969(pJRC200) is specifically caused by trans-complementation by the wild-type *cpe* gene present in pJRC200.

Expression of α*-toxin by* cpe *knock-out mutants*

To confirm that the *cpe* mutation introduced into MRS101 and MRS4969 was specific for CPE expression and had

Table 2. Fluid accumulation of rabbit ileal loops injected with *C. perfringens* lysates.

Culture	Medium[a]	Lysate concentration[b,c]	No. positive/ no. tested[d]	V/L[e]	Death
F4969	DS	50×	2/2	0.9 ± 0.3	All
	DS	25×	2/2	1.6 ± 0.3	All
	DS	10×	3/3	1.1 ± 0.3	None
	FTG	50×	0/5	<0.1	None
MRS4969	DS	50×	0/5	<0.1	None
	FTG	50×	0/5	<0.1	None
MRS4969(pJRC200)	DS	50×	3/3	0.6 ± 0.2	All
	DS	10×	3/3	1.3 ± 0.4	None
	FTG	50×	0/5	<0.1	None
MRS4969(pJIR418)	DS	50×	0/2	<0.1	None
	FTG	50×	0/2	<0.1	None
SM101	DS	50×	3/3	1.6 ± 0.1	None
	DS	25×	1/1	0.4	None
	FTG	50×	0/4	<0.1	None
MRS101	DS	50×	0/5	<0.1	None
	FTG	50×	0/3	<0.1	None
MRS101(pJRC200)	DS	50×	4/4	1.3 ± 0.4	None
	FTG	50×	0/4	<0.1	None
MRS101(pJIR418)	DS	50×	0/2	<0.1	None
	FTG	50×	0/2	≤0.1	None

a. DS stands for cultures grown in Duncan–Strong sporulation medium, and FTG for cultures grown in fluid thioglycollate medium (which supports only vegetative growth).
b. After concentration, lysates were always analysed by CPE Western blots. That analysis confirmed that, when corrected for concentration, CPE levels in concentrated DS lysates were very similar to those shown for unconcentrated DS lysates in Table 1. No CPE expression was detected in any (even 50-fold) concentrated FTG lysate.
c. Microscopic counting of FTG and DS cultures confirmed that the number of vegetative and sporulating bacteria present in each culture, before concentration, was highly reproducible for each independently prepared sample (data not shown), and that no significant difference in total growth or sporulation existed between a parent and its derivatives (Table 1 and data not shown).
d. Each ileal loop received an independently grown and concentrated culture lysate.
e. V/L, ratio of ileal loop volume to length.

not affected global toxin production, wild-type (SM101 and F4969) and mutant (MRS101 and MRS4969) strains were tested for their α-toxin-producing abilities. Using the reverse CAMP test (Hansen and Elliott, 1980), all wild-type and mutant isolates were found to produce (data not shown) the arrow-shaped zone of synergistic haemolysis that is indicative of α-toxin expression.

In vivo *virulence testing of culture lysates of* cpe *knock-out mutants and derivatives*

The gastrointestinal virulence of concentrated culture lysates of wild-type, mutant and complementing strains were compared in a rabbit ileal loop model, which is the standard model for evaluating *C. perfringens* enterotoxicity (Duncan and Strong, 1969). Our experiments specifically assessed the ability of these concentrated culture lysates to induce fluid accumulation or histopathological damage in rabbit ileal loops.

Ileal loop fluid accumulation. Initial control experiments confirmed that neither 50-fold-concentrated FTG broth nor 50-fold-concentrated DS medium induced ileal loop fluid accumulation (data not shown). Similarly, no ileal loop fluid accumulation was detected using even 50-fold-concentrated vegetative culture lysates of wild-type strains SM101 or F4969. However, 50-fold-concentrated lysates prepared from sporulating cultures of SM101 or F4969 reproducibly induced ileal loop fluid accumulation (Table 2); in the case of F4969, 50-fold or 25-fold-concentrated sporulating culture lysates also induced rabbit death (Table 2), while 10-fold-concentrated sporulating culture lysates of this wild-type strain induced fluid accumulation without rabbit death.

In contrast to the results reported above for sporulating culture lysates of the wild-type parent isolates, 50-fold-concentrated lysates prepared from either vegetative or sporulating cultures of MRS101 or MRS4969 failed to induce any ileal loop fluid accumulation (Table 2). To confirm that the failure of these concentrated sporulating culture lysates to induce ileal loop effects was not attributable to polar mutations, similar ileal loop experiments were conducted using concentrated culture lysates of MRS101(pJRC200) and MRS4969(pJRC200). While no ileal loop fluid accumulation was observed using even 50-fold-concentrated vegetative culture lysates of either

pJRC200-complemented mutant, 50-fold-concentrated lysates prepared from sporulating cultures of both MRS101(pJRC200) and MRS4969(pJRC200) reproducibly induced ileal loop fluid accumulation (Table 2) and, in the case of MRS4969(pJRC200), rabbit death. Furthermore, even 10-fold-concentrated sporulating culture lysates of MRS4969(pJRC200) reproducibly stimulated fluid accumulation, although these lysates did not cause death. Consistent with the intestinal effects of these pJRC200-complemented mutants being caused specifically by the *cpe* gene present in pJRC200, neither fluid accumulation nor death was observed (Table 2) using even 50-fold-concentrated lysates prepared from either sporulating or vegetative cultures of MRS101(pJIR418) or MRS4969(pJIR418).

Histological damage induced by cpe *mutants and derivatives*. Previous studies (Duncan *et al*., 1968; Niilo, 1970) have demonstrated that sporulating culture lysates of *cpe*-positive *C. perfringens* type A isolates can induce histopathological damage in rabbit ileal loops. To investigate the relative contribution of CPE to this histopathological damage, blinded histological analysis was performed on tissue samples removed from ileal loops treated with concentrated vegetative or sporulating culture lysates of SM101 or F4969, *cpe* knock-out mutants MRS101 or MRS4969, or complementing strains MRS101(pJRC200) or MRS4969(pJRC200).

Tissue specimens from ileal loops treated with vegetative culture lysates of wild-type strains SM101 or F4969 were indistinguishable from normal small intestinal mucosa (data not shown), i.e. these specimens showed no desquamation of the epithelium, maintained a villi–crypt ratio of 4–5:1 and exhibited virtually no polymorphonuclear leukocyte (PMN) infiltration (Fig. 4). However, significant histological damage was observed reproducibly in ileal loops

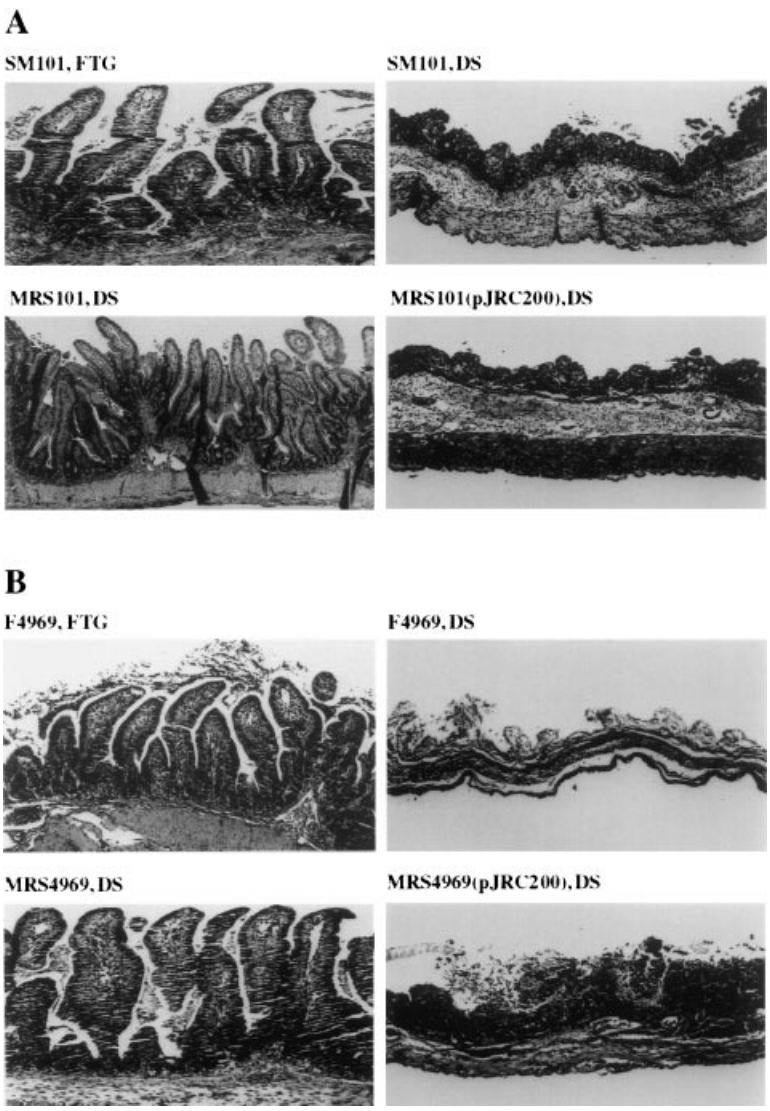

Fig. 4. Histological damage induced by *C. perfringens* lysates. Tissue specimens shown were collected from rabbit ileal loops treated with either concentrated vegetative (FTG) or concentrated sporulating (DS) culture lysates of *C. perfringens* wild-type, mutant or complementing strains.
A. Tissue specimens shown were treated with 50-fold-concentrated DS or FTG (as indicated) lysates of wild-type SM101, *cpe* knock-out mutant MRS101 or complementing strain MRS101(pJRC200). Tissue specimens treated with 50-fold-concentrated FTG lysates prepared from either MRS101 or complementing strain MRS101(pJRC200) were indistinguishable from specimens treated with FTG lysates of SM101 (data not shown).
B. Tissue specimens treated with 50-fold-concentrated FTG lysates of wild-type F4969, 10-fold-concentrated DS lysates of wild-type F4969, 50-fold-concentrated DS lysates of *cpe* knock-out mutant MRS4969 or 10-fold-concentrated DS lysates of complementing strain MRS4969(pJRC200). Tissue specimens treated with 50-fold-concentrated FTG lysates prepared from either MRS4969 or complementing strain MRS4969(pJRC200) were indistinguishable from specimens treated with 50-fold-concentrated FTG lysates of F4969 (data not shown). All specimens are shown at 250× magnification.

treated with sporulating culture lysates of wild-type strains SM101 or F4969 (Fig. 4). Similar to previous observations using purified CPE (McDonel and Duncan, 1975; Sherman *et al*., 1994), this damage most prominently included severe desquamation of the villus epithelium, shortening of villi (producing a villus–crypt ratio of $\leq$1:1) and transmural infiltration by PMNs. These results confirm that expression of a sporulation-associated factor is responsible for histopathological damage induced by SM101 and F4969.

However, histopathological damage was absent from ileal loops treated with sporulating culture lysates of *cpe* knock-out mutants MRS101 or MRS4969 (Fig. 4), i.e. these specimens exhibited no desquamation, maintained a villi–crypt ratio of 4–5:1 and exhibited no PMN infiltration. These results implicate CPE as the active, sporulation-associated factor responsible for the histological damage caused by sporulating culture lysates of SM101 and F4969. This contention receives further support from the observed ability of sporulating culture lysates of complementing strains MRS101(pJRC200) or MRS4649(pJRC200) to induce histopathological damage indistinguishable from that caused by sporulating culture lysates of wild-type isolates SM101 or F4969 (Fig. 4).

Discussion

While CPE-producing strains of *C. perfringens* type A have been implicated for at least 15 years as important causes of food-borne and non-food-borne GI diseases of humans, the pathogenesis of these illnesses remains incompletely understood. Previous studies (McDonel and Duncan, 1977; Sherman *et al*., 1994) have shown that rabbit ileal loops treated with highly purified CPE accumulate significant amounts of luminal fluid and develop extensive intestinal histopathological damage. Furthermore, human volunteer feeding experiments (Skjelkvale and Uemura, 1977) have demonstrated that ingestion of highly purified CPE reproduces the abdominal cramping and diarrhoeal symptoms that characterize the GI illnesses associated with *cpe*-positive *C. perfringens* type A isolates. Collectively, these previous results strongly suggest that CPE expression is sufficient for a *cpe*-positive *C. perfringens* type A isolate to induce GI effects. However, given that (i) *cpe*-positive *C. perfringens* type A isolates also typically express alpha toxin; (ii) at least some variants of alpha toxin, which is produced by type A isolates, have been implicated in some GI diseases of domestic animals (Ginter *et al*., 1996); and (iii) *cpe*-positive *C. perfringens* type A isolates remain poorly characterized for their ability to produce minor or unrecognized toxins, it has been difficult to evaluate whether CPE expression is necessary for *cpe*-positive *C. perfringens* type A disease isolates to cause GI effects.

In the current study, we specifically inactivated the *cpe* gene in SM101, a transformable derivative of a *cpe*-positive *C. perfringens* type A food poisoning isolate that carries a chromosomal *cpe* gene, and F4969, a *cpe*-positive type A non-food-borne human GI disease isolate that carries a plasmid-borne *cpe* gene. These mutations dramatically affected the GI virulence of these two human GI disease isolates in a concentrated lysate:rabbit ileal loop model. Among the phenotypic consequences of inactivating the *cpe* gene was the complete loss of ability of sporulating culture lysates prepared from either mutant to induce fluid accumulation in rabbit ileal loops. The reversal of this effect, noted when the *cpe* mutation was complemented by a recombinant plasmid carrying the wild-type *cpe* gene, allows us specifically to assign CPE as the virulence factor responsible for all ileal loop fluid accumulation induced by sporulating culture lysates prepared from both the SM101 and the F4969 wild-type disease isolates. Given the strong association between CPE expression and sporulation, our observation that ileal loop fluid accumulation was only induced by lysates prepared from sporulating cultures of the wild-type or complementing strains provides additional (although indirect) evidence that CPE is the toxin responsible for the ileal loop fluid accumulation induced by these two disease isolates. Furthermore, our results also indicate that CPE expression is necessary for the intestinal histopathological damage induced by F4969 and SM101. This claim is supported by the failure of lysates prepared from our *cpe* knock-out mutants to produce any histopathological damage in ileal loops and by the restoration of this damage using lysates prepared from these mutants complemented with a recombinant plasmid carrying the wild-type *cpe* gene. Additional indirect support for this claim includes our observation that lysates of wild-type or complementing strains only caused histological damage when prepared from cultures grown under the sporulating conditions known to induce CPE expression.

Collating our current results with those of previous studies (McDonel and Duncan, 1977; Sherman *et al*., 1994), it can now be concluded that CPE expression appears to be both necessary and sufficient for *C. perfringens* type A disease isolates SM101 and F4969 to induce fluid accumulation and histopathological damage in the concentrated lysate: rabbit ileal loop model. By extension, these results suggest that CPE is an important, if not essential, virulence factor for the GI effects induced by other *cpe*-positive *C. perfringens* type A isolates during human disease. However, our results obviously do not preclude the possibility that other toxins sometimes contribute to the GI pathogenesis of *cpe*-positive *C. perfringens* type A isolates. For example, it remains conceivable that the human GI tract is more sensitive than the rabbit GI tract to some other *C. perfringens* toxin(s), that other *cpe*-positive *C. perfringens* isolates might express additional toxins not produced by SM101 or

F4969, or that another *C. perfringens* toxin with GI activity is made by SM101 or F4969, but is only expressed *in vivo*.

As in past studies evaluating the enterotoxicity of *C. perfringens* isolates (Duncan and Strong, 1969), we concentrated *C. perfringens* culture lysates before adding these samples to ileal loops. The rationale for using concentrated lysates in ileal loop studies is to minimize sample volumes injected into ileal loops, which makes detection of mild fluid accumulation possible. Interestingly, we observed that the addition of highly (25- or 50-fold) concentrated lysates prepared from sporulating, but not vegetative, cultures of F4969 reproducibly induced the death of rabbits. This effect was absent using sporulating (or vegetative) culture lysates prepared from the *cpe* knock-out mutant MRS4969, although lethality was restored using 25- or 50-fold-concentrated sporulating culture lysates prepared from MRS4969 complemented with a plasmid carrying a wild-type *cpe* gene. Collectively, these observations identify CPE as the lethal factor in lysates prepared from sporulating cultures of F4969. It is conceivable that similar lethal concentrations of CPE might sometimes accumulate in the human intestines during GI disease involving *cpe*-positive *C. perfringens* type A isolates and that this effect might explain the human fatalities that occasionally occur during CPE-associated human GI illnesses (McClane, 1997).

Interestingly, when 50-fold-concentrated sporulating cultures of the CPE-positive wild-type isolate SM101 were injected into rabbit ileal loops, no lethality was observed. As previous studies (Collie *et al*., 1998) have indicated that the *cpe* open reading frame (ORF) is highly conserved between *cpe*-positive isolates and that the CPE expressed by different *cpe*-positive isolates exhibits similar cytotoxic activity, the most likely explanation for the observed ability of 50-fold-concentrated lysates of sporulating cultures of F4969, but not of SM101, to induce lethality when added to the lumen of rabbit ileal loops is the difference in CPE expression levels detected in our studies between these two CPE-positive isolates, i.e. in Table 1, we measured ~fivefold higher CPE expression by sporulating cultures of F4969 versus SM101. Also supporting this explanation, Table 2 shows that lysates prepared from 10-fold-concentrated sporulating cultures of F4969 were non-lethal.

The differences in CPE expression levels noted between sporulating cultures of SM101 and F4969 are consistent with other studies noting significant variations in CPE expression levels among *cpe*-positive isolates (Collie *et al*., 1998). Those previous studies indicated that CPE expression differences do not correlate with a chromosomal versus plasmid *cpe* gene location. Differences in CPE expression are also not explainable by a gene dosage effect, because (as our Southern blot and PCR results strongly suggest) SM101 and F4969 both appear to carry a single copy of the *cpe* gene. Given the strong association between CPE expression and sporulation, differences in sporulation ability probably help to explain isolate-to-isolate variations in CPE expression. Consistent with this hypothesis, our Table 1 results show that F4969 sporulates better than SM101 under the growth conditions used in our studies. However, differences in sporulation probably do not completely explain CPE expression differences between F4969 and SM101, as F4969 was ~1.7-fold better at sporulating than SM101, but ~five-fold better at producing CPE. Therefore, other regulatory differences apparently also exist between SM101 and F4969; in future studies, we hope to explore these regulatory differences in CPE expression.

Finally, our report represents (to our knowledge) only the second successful study constructing clostridial toxin gene knock-outs using double reciprocal cross-over events. A seminal previous study (Awad *et al*., 1995) has reported the successful construction of knock-out mutants of *pfoA* and *plc*, the chromosomal genes encoding *C. perfringens* theta toxin (perfringolysin O) and alpha toxin respectively. It should be noted that constructing *cpe* knock-outs involved several additional challenges beyond those encountered during the construction of the *pfoA* and *plc* knock-out mutants. First, to be useful for virulence studies, our *cpe* knock-out mutants needed to retain their ability to sporulate at levels similar to their wild-type parents. Retaining sporulation in our mutants was not a trivial concern, given the heterogeneity in sporulation capability existing even within a culture of a single *C. perfringens* isolate. Another challenge was that, because it is a *cpe*-negative strain, we could not mutate the *C. perfringens* strain 13 used to construct the *pfoA* and *plc* knock-outs. Our inability to use strain 13 was unfortunate, because that strain is much more susceptible to electroporation than other *C. perfringens* isolates. Consequently, we expended considerable effort (i) identifying wild-type *cpe*-positive strains susceptible, at any frequency, to electroporation; and (ii) introducing our suicidal plasmid into these isolates, which was made more difficult by the low frequency of electroporation of SM101 and F4969. However, the greatest challenge faced in our studies was the lack of an easy selection for *cpe*, a gene whose inactivation does not produce an easily screened phenotype (the previous study constructing *pfoA* and *plc* knock-outs could identify those toxin mutants simply by plating transformants on blood agar or egg yolk agar plates and observing transformants for loss of haemolytic or phospholipase C activity respectively). To overcome this screening problem, we constructed a mutator plasmid that contains two antibiotic resistance markers, one for the selection of the mutant allele and the other for vector DNA. This approach, which involves selecting double cross-over events by monitoring for a chloramphenicol-resistant, but erythromycin-sensitive, phenotype should have widespread applicability for conducting

other virulence studies in *C. perfringens* (and perhaps other clostridial species).

Experimental procedures

Bacteria and growth conditions

All bacteria and plasmids used in this study are described in Table 3. *E. coli* DH5α cultures were grown overnight, with shaking, at 37°C in TSB (Difco) medium. Vegetative *C. perfringens* cultures were grown for 9 h at 37°C in FTG (Difco) broth. Duncan–Strong medium (Kokai-kun *et al*., 1994) was used to obtain sporulating cultures of *C. perfringens*. DS cultures of *C. perfringens* were grown for 8 h at 37°C. All media were supplemented with 20 μg ml^{-1} chloramphenicol or 50 μg ml^{-1} erythromycin for growing, respectively, knockout mutants or transformants containing pJIR418-based constructs.

Construction of cpe *mutator plasmid pMRS103*

Plasmid pJRC100, a derivative of the *E. coli–C. perfringens* shuttle vector pJIR418 (Czeczulin *et al*., 1996), contains a 5.7 kb *Xba*I *C. perfringens* DNA insert, which includes the intact *cpe* gene; this plasmid also has erythromycin and chloramphenicol resistance genes (*ermBP* and *catP* respectively), as well as *oriCP* and *oriEC* (origins of replication for *C. perfringens* and *E. coli* respectively). The *catP* and *oriCP* genes of pJRC100 were deleted by digesting this plasmid with *Fsp*I and *Eco*RV, forming pMRS102. The *cat*P gene was then reinserted into the *Bgl*II–*Nhe*I sites located within the *cpe* gene of pMRS102 by (i) digesting pMRS102 with *Bgl*II and *Nhe*I, which removes a 0.4 kb internal *cpe* fragment; (ii) filling in the resultant *Bgl*II–*Nhe*I ends by the Klenow reaction; and (iii) blunt end ligating a *Sma*I–*Nae*I fragment, which contains the *catP* gene from pJRC100, to form pMRS103. This new plasmid contains an inactive *cpe* gene and, as it contains no origin of replication for *C. perfringens* (*oriCP*), is suicidal for *C. perfringens*.

Isolation of cpe *knock-out mutants*

The plasmid pMRS103 was used to transform, by electroporation (Czeczulin *et al*., 1996), *C. perfringens* isolates SM101 and F4969 to erythromycin (50 μg ml^{-1}) and chloramphenicol (20 μg ml^{-1}) resistance. One transformant with each resistance was then grown overnight at 37°C in TGY broth (3% trypticase, 2% glucose, 1% yeast extract and 0.1% cysteine) containing erythromycin (50 μg ml^{-1}) and chloramphenicol (20 μg ml^{-1}). A 200 μl aliquot of that culture was inoculated into TGY broth without any antibiotics and allowed to grow overnight at 37°C. A 100 μl aliquot of a 10^{-5} dilution of this overnight culture was spread on BHI plates, which were then incubated overnight at 37°C under anaerobic conditions. Single colonies were patched onto a fresh BHI plate containing erythromycin (50 μg ml^{-1}) or chloramphenicol (20 μg ml^{-1}). After 24 h incubation at 37°C, colonies sensitive to erythromycin, but resistant to chloramphenicol, were selected. After the presence of mutant *cpe* alleles was confirmed by PCR (see *Results*), these isogenic mutant derivatives of SM101 and F4969 were designated MRS101 and MRS4969 respectively.

Preparation of digoxigenin-labelled probes

cpe *probe*. A 639 bp digoxigenin (DIG)-labelled, double-stranded, *cpe*-specific DNA gene probe was prepared by a previously described, two-step PCR amplification method

Table 3. Bacterial strains and plasmids used in this study.

Strain or plasmid	Relevant characteristics	Source or reference
C. perfringens		
F4969	Non-food-borne disease type A isolate, carries plasmid-borne *cpe* gene	M. Brett
SM101	Food poisoning type A isolate, carries chromosomal *cpe* gene	Zhao and Melville (1998)
E. coli		
DH5α		BRL Laboratories
Plasmids		
pJIR418	*C. perfringens/E. coli* shuttle plasmid; Cm^R, Em^R	Sloan *et al*. (1992)
pJRC100	5.7 kb *Xba*I *C. perfringens* DNA fragment, which contains the *cpe* ORF, ligated into pJIR418	Czeczulin *et al*. (1996)
pJRC200	1.6 kb PCR fragment, which contains only the *cpe* ORF, inserted into pJIR418	Czeczulin *et al*. (1996)
pMRS102	Prepared by deletion of 5.5 kb *Fsp*I–*Eco*RV fragment, which contains *catP* and *oriCP*, from pJRC100	This work
pMRS103	0.4 kb *Bgl*II–*Nhe*I fragment from *cpe* ORF in pMRS102 was replaced with a 1.3 kb *Sma*I-*Nae*I fragment, which contains chloramphenicol determinant (*catP*), from pJRC100	This work

(Czeczulin *et al*., 1996), using the primer set 5′-GGTACCTT-TAGCCAATCA-3′ (primer 2F) and 5′-TCCATCACCTAAG-GACTG-3′ (primer 5R).

catP *probe*. A 517 bp DNA fragment containing internal *catP* gene sequences was extracted from pMRS103 by digesting this plasmid with *Eco*RV and *Hpa*I. This DNA fragment was then gel purified and labelled using a random-primed DNA labelling system, as described in *The Genius System Users Guide for Filter Hybridization* (Boehringer Mannheim).

pJIR418 vector probe. About 2.4 kb of pJIR418 vector DNA sequences present in pMRS103 was extracted by digesting pMRS103 with *Sma*I. These DNA sequences were then labelled using a random-primed DNA labelling system, as described in *The Genius System Users Guide for Filter Hybridization* (Boehringer Mannheim).

Southern blot analysis

A previously described protocol (Czeczulin *et al*., 1996) was used, with modification, to isolate total *C. perfringens* DNA for Southern blot experiments. DNA samples were digested to completion with *Hpa*I, separated by electrophoresis on 0.8% agarose gels, transferred to positively charged nylon membranes (Boehringer Mannheim) and then UV-fixed to these membranes. Dig-labelled gene probes, prepared as indicated above, were hybridized to these blots as described in *The Genius System Users Guide for Filter Hybridization* (Boehringer Mannheim). Hybridized probe was detected using a DIG-chemiluminescence detection system with CSPD substrate (Boehringer Mannheim).

Standard DNA modification techniques

Plasmid DNA isolation, restriction enzyme digestion, ligation and transformation was carried out using standard methods (Sambrook *et al*., 1989).

Western blot analysis

C. perfringens cultures were grown at 37°C for 8 h in DS, or for 9 h in FTG, and then sonicated until > 95% of all cells were lysed (lysis progress was monitored continuously by phase-contrast microscopy). After sonication, each culture lysate was analysed for the presence of CPE using Western immunoblots (Kokai-Kun *et al*., 1994; Czeczulin *et al*., 1996; Collie *et al*., 1998), and the amount of CPE present in these CPE-positive lysates was quantified by densitometric comparisons against a dilution series (20–100 ng lane^{-1}) of purified CPE, as described previously (Czeczulin *et al*., 1996; Collie *et al*., 1998).

Determination of vegetative and sporulating cell numbers in C. perfringens *cultures*

The numbers of vegetative cells and refractile endospores (i.e. sporulating cells) present per millilitre of sporulating *C. perfringens* culture were determined microscopically using a Hawksley counting chamber, as described previously (Czeczulin *et al*., 1996; Collie *et al*., 1998). At least 64 squares (enough squares were counted to ensure that at least 600 cells were counted for every sample) of the counting chamber were examined with a phase-contrast microscope (Nikon). Values shown represent means from at least three independent growths of each strain or transformant.

Preparation of C. perfringens *cell lysates for ileal loop experiments*

C. perfringens cultures (500 ml) were grown at 37°C for 9 h in FTG or for 8 h in DS. Culture supernatants were initially separated from cell pellets by centrifugation and, unless otherwise specified, concentrated to 10 ml using an Amicon PM 10 concentrator. The cell pellets were then resuspended with this concentrated supernatant (at the same ratio as present in the original culture), and the mixture was sonicated until > 95% of all cells were lysed (lysis progress was monitored continuously by phase-contrast microscopy). After sonication, the lysate was centrifuged, and the lysate supernatants were quantified for the presence of CPE using Western immunoblotting (Czeczulin *et al*., 1996; Collie *et al*., 1998). Culture lysates were stored at −70°C until use.

Each ileal loop sample tested in Table 2 and Fig. 4 was prepared from an independently grown culture. Bacterial numbers (total, sporulating and vegetative cells ml^{-1}) and CPE expression levels (μg ml^{-1}) were determined, before concentration, for every independent DS or FTG culture by microscopic counting and Western immunoblots respectively. Total growth, sporulation and CPE expression levels were found to be highly reproducible (data not shown) for independent DS or FTG cultures of a wild-type parent strain or its derivatives. Furthermore, Western blot analysis of concentrated lysates confirmed that appreciable amounts of CPE were not lost during the concentration process (data not shown).

Ileal loop assay for enterotoxic activity

Enterotoxic activity in culture lysates of *C. perfringens* mutants and derivatives was assessed using the standard method for evaluating the enterotoxic properties of *C. perfringens* isolates (Duncan and Strong, 1969). New Zealand White rabbits (of either sex, > 12 weeks of age) were fasted overnight and then anaesthetized with 0.25 ml of ketamine hydrochloride (100 mg ml^{-1}) mixed with 0.25 ml of diazepam (5 mg ml^{-1}) injected intravenously in the marginal ear vein. Anaesthesia was maintained using halothane (1.5–2.5% to effect), nitrous oxide (2 l min^{-1} flow) and oxygen (1 l min^{-1}) delivered via a gas anaesthesia machine. The mid-section of each anaesthetized rabbit's abdomen was shaved and prepared aseptically using a series of alternating betadyne and isopropyl alcohol scrubs, and a 5 cm abdominal incision was made. The ileum was then carefully withdrawn, and ileal loops (7–10 cm long) were constructed by sealing a section of ileum at its distal end with two sterile cotton ligatures, 1 cm apart, and a single ligature at the other. Fluid (0.5 ml loop^{-1}) was injected through a 26-gauge needle into each loop at a location about 0.5 cm immediately below the single proximal ligature. The injection site was isolated to prevent leakage by a further ligature about 0.5 cm below the puncture, making a pair of ligatures at either end of

each loop. In each animal, seven loops were prepared; test fluids were introduced into loops 1, 3, 5 and 7, while loops 2, 4 and 6 were left empty to control for any fluid response caused by the procedure, rather than the nature of the inocula. This control response was seen only once, and the material in that blank loop was readily distinguished from that in positive test loops by its dark colour, its thicker than water consistency and the presence of ingesta. After inoculation of the loops, the ileum was again moistened with warm saline and gently returned to the abdominal cavity. After suturing the muscle wall and closing the skin incision, the animals were kept warm and monitored during the anaesthetic recovery period. Oxymorphone (0.25 ml i.m./rabbit; 1.5 mg ml^{-1}) was given before anaesthetic recovery and again at 6–8 h after surgery. Food and water were withheld post-operatively.

Approximately 16–18 h after surgery, the rabbits were euthanized with a 0.5–1.0 ml intravenous injection of Beuthanasia D (390 mg ml^{-1} pentobarbital, 50 mg ml^{-1} phenytoin). The ileum was removed, and fluid accumulation in individual loops was assessed visually; the length of each positive loop was then measured and its contents weighed for calculating the V/L (volume–length) ratio, which is the ratio of weight of loop contents in grams to loop length in centimetres. Positive loops (those accumulating fluid) had V/L ratios > 0.3 and contained a serosanguinous fluid with a free-flowing, watery consistency. Negative loops had no recoverable content, i.e. their V/L ratios were < 0.1.

The use of this rabbit ileal loop assay was approved by the Virginia Tech Animal Care Committee, and all animal care and experimental procedures were conducted in compliance with pertinent federal, state and local legislation and guidelines.

Histology

At autopsy, tissues were collected and stored in 10% neutral-buffered formalin. For subsequent histological analysis, these preserved tissues were paraffin embedded, thin sectioned, stained with haemotoxylin and eosin, and viewed microscopically by a veterinary pathologist who was blinded to the origin of each specimen.

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