

Enterotoxin Plasmid from *Clostridium perfringens* Is Conjugative

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Clostridium perfringens enterotoxin is the major virulence factor involved in the pathogenesis of *C. perfringens* type A food poisoning and several non-food-borne human gastrointestinal illnesses. The enterotoxin gene, *cpe*, is located on the chromosome of food-poisoning isolates but is found on a large plasmid in non-food-borne gastrointestinal disease isolates and in veterinary isolates. To evaluate whether the *cpe* plasmid encodes its own conjugative transfer, a *C. perfringens* strain carrying pMRS4969, a plasmid in which a 0.4-kb segment internal to the *cpe* gene had been replaced by the chloramphenicol resistance gene *catP*, was used as a donor in matings with several *cpe*-negative *C. perfringens* isolates. Chloramphenicol resistance was transferred at frequencies ranging from 2.0×10^{-2} to 4.6×10^{-4} transconjugants per donor cell. The transconjugants were characterized by PCR, pulsed-field gel electrophoresis, and Southern hybridization analyses. The results demonstrated that the entire pMRS4969 plasmid had been transferred to the recipient strain. Plasmid transfer required cell-to-cell contact and was DNase resistant, indicating that transfer occurred by a conjugation mechanism. In addition, several fragments of the prototype *C. perfringens* tetracycline resistance plasmid, pCW3, hybridized with pMRS4969, suggesting that pCW3 shares some similarity to pMRS4969. The clinical significance of these findings is that if conjugative transfer of the *cpe* plasmid occurred in vivo, it would have the potential to convert *cpe*-negative *C. perfringens* strains in normal intestinal flora into strains capable of causing gastrointestinal disease.

Clostridium perfringens is an important cause of enteric and histotoxic infections in both humans and animals and produces several potent toxins (22). The alpha-toxin structural gene is invariably located on the chromosome (10), but the genes encoding the beta-, epsilon-, and iota-toxins are located on large plasmids (10, 21).

C. perfringens enterotoxin (CPE) ranks among the most medically important *C. perfringens* toxins. Although representing only less than 5% of all *C. perfringens* isolates, CPE-positive type A strains are significant enteric pathogens. In humans, they cause *C. perfringens* type A food poisoning, which is one of the most common food-borne illnesses in developed countries, and 5 to 20% of all cases of non-food-borne human gastrointestinal (GI) illness, including antibiotic-associated diarrhea and sporadic diarrhea (12, 19). Recent studies (24) have demonstrated that CPE expression is necessary for the pathogenesis of both *C. perfringens* type A food poisoning and non-food-borne GI disease isolates. In those studies, isogenic *cpe* knockout mutants were prepared from SM101, a transformable derivative of the food poisoning isolate NCTC8798, which carries a chromosomal *cpe* gene, and F4969, a non-food-borne GI disease isolate carrying a plasmid *cpe* gene. Both mutants were constructed by replacing a 0.4-kb segment internal to the *cpe* gene with the chloramphenicol resistance gene *catP*. Although sporulating, but not vegetative, culture lysates of both wild-type parents induced significant rabbit ileal loop

fluid accumulation and histopathologic damage, neither sporulating nor vegetative culture lysates of the *cpe* knockout mutants induced either effect. However, virulence was restored when these mutants were complemented with a recombinant plasmid carrying the wild-type *cpe* gene, confirming that loss of virulence can be specifically attributed to inactivation of the *cpe* gene and the resultant loss of sporulation-associated CPE production (24).

In human food poisoning isolates, the chromosomal *cpe* gene is located on what appears to be a transposable genetic element, Tn5565 (8, 9). In contrast, non-food-borne human GI disease isolates and animal isolates carry the *cpe* gene on large plasmids (11, 13, 27). These plasmids have not yet been fully characterized but appear to vary somewhat in size (average, ≈ 100 kb) and carry IS elements. Other *C. perfringens* toxin types occasionally carry *cpe* plasmids, which may have both IS elements and genes encoding other toxins (15).

There is circumstantial evidence suggesting that the large *cpe* plasmids are conjugative. Firstly, type A strains carrying the *cpe* plasmid do not share the same genetic background (12). Secondly, several nonclonal type E isolates have been found to carry a plasmid with a defective but highly conserved copy of the *cpe* gene as well as the iota-toxin genes *iap* and *ibp* (5). The objective of this study was to test the hypothesis that the *cpe* plasmids are conjugative.

The *cpe*Ω*catP* plasmid pMRS4969 is conjugative. Our approach was to use mixed plate matings to transfer pMRS4969, a plasmid that carries a *cpe* gene inactivated by deletion of internal *cpe* sequences and insertion of the *catP* gene (24). To monitor the transfer of the *cpe* plasmid, *C. perfringens* strain MRS4969, which carries the *cpe*Ω*catP* plasmid pMRS4969, was initially used as a donor in mixed plate matings (23) with

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TABLE 1. *C. perfringens* strains

Strain	Description ^a	Reference or source
F4969	Wild-type CPE ⁺	24
MRS4969	F4969(pMRS4969) Cm ^r	24
13	Wild type	18
CW362	Wild type	23
CW504	CW362 Rif ^r Nal ^r	23
JIR39	CW362 Chl ^r Sm ^r	20
JIR100	Em ^r	4
JIR325	13 Rif ^r Nal ^r	17
JIR458	13 Sm ^r	T. Bannam and J. Rood
JIR4468	JIR325(pMRS4969) Cm ^r	Transformant
JIR4475	JIR458(pMRS4969) Cm ^r	Transconjugant, JIR4468 × JIR458
JIR4479	JIR325(pMRS4969) Cm ^r	Transconjugant, JIR4475 × JIR325
JIR4481	JIR39(pMRS4969) Cm ^r	Transconjugant, JIR4468 × JIR39

^a Rif, rifampin; Nal, nalidixic acid; Chl, potassium chlorate; Sm, streptomycin; Cm, chloramphenicol; Em, erythromycin.

several genetically marked *C. perfringens* recipient strains. The *C. perfringens* strains (Table 1) were cultured in various media, as previously described (20, 23). When required, appropriate concentrations of rifampin (1 or 20 µg/ml), nalidixic acid (20 µg/ml), chloramphenicol (5 or 15 µg/ml), or streptomycin (1,000 µg/ml) were added to the medium. Agar plates were grown in a 10% H₂-10% CO₂-80% N₂ atmosphere. For the matings, single colonies of the donor and recipient strains were grown to exponential phase in fluid thioglycollate broth and 100 µl of each culture was mixed and spread on the surface of a nonselective agar plate. After incubation overnight, the cells were resuspended in 3 ml of brain heart infusion broth and 10-fold dilutions were plated onto the appropriate media for selection of the transconjugants.

Unfortunately, MRS4969 produced a substance, presumably a bacteriocin, that killed or inhibited the growth of the poten-

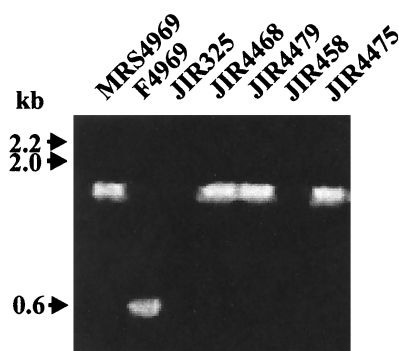


FIG. 1. PCR analysis of transconjugants. Fresh cells from agar plates were resuspended in 100 µl of H₂O and lysed in a microwave oven for 3 min. Three microliters of the supernatant was used in a standard PCR (50-µl volume) in a DNA thermal cycler (Perkin-Elmer) with 30 cycles of 30 s at 94°C, 30 s at 50°C, and 90 s at 72°C. The primers were cpeR (5'-CATCACCTAAGGACTGTTCT-3') and cpeF (5'-TGTAGAATATGGATTTGGAAT-3'). The resultant products were separated by agarose gel electrophoresis. As shown, wild-type *cpe*-positive cells yielded a *cpe* band with a size of 544 bp, whereas a 1.7-kb band was present in strains with the *cpeΩcatP* region. Molecular sizes of the DNA markers are shown on the left.

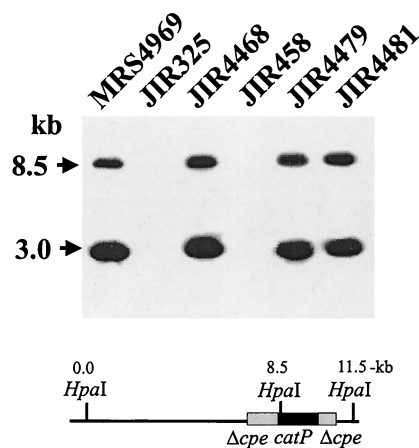


FIG. 2. Southern blot of parent strains and transconjugants. *C. perfringens* DNA was isolated as described previously (14), digested with *Hpa*I, separated by electrophoresis on a 1% agarose gel, and transferred by Southern blotting to positively charged nylon membranes (Roche Molecular Biochemicals). The membrane was then hybridized at 68°C overnight with a digoxigenin-labeled *cpe* probe. To prepare the probe, an ≈1.6-kb fragment containing the *cpe* gene and the regions ≈300-bp upstream and ≈200-bp downstream, was gel purified from *Eco*RI- and *Xba*I-digested pJRC200 DNA (14) and labeled by using a random-primed DNA labeling system (Boehringer Mannheim). After hybridization, the membrane was washed twice for 15 min each in wash solution (2× SSC [1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate]-0.1% sodium dodecyl sulfate) at room temperature. The final washes were twice for 15 min each in 0.5× wash solution (0.5× SSC-0.1% sodium dodecyl sulfate) at 68°C. Hybridized probe was then detected by using a digoxigenin-chemiluminescence detection system with CSPD substrate (Roche Molecular Biochemicals). Molecular sizes of the DNA markers are shown on the left. The map shows the relevant *cpeΩcatP* region of pMRS4969.

tial recipients. Therefore, no transconjugants were obtained in these matings. To move the plasmid into a cleaner background, pMRS4969 was purified from MRS4969 using a cesium chloride-ethidium bromide density gradient (1), without NaCl treatment of the lysate, and used to transform the *cpe*-negative strain JIR325 to chloramphenicol resistance. Transformation of *C. perfringens* cells was carried out by electroporation (26) in 0.2-cm Gene Pulser cuvettes (Bio-Rad) at 2.5 kV, 25 µF, and 200 Ω. JIR325 is a rifampin- and nalidixic acid-resistant derivative of strain 13, the transformable *C. perfringens* strain most commonly used in genetic studies (17). The presence of the *cpeΩcatP* gene region in the resultant transformant, JIR4468, was confirmed by PCR (Fig. 1) and Southern hybridization analyses (Fig. 2).

When JIR4468 was mated with JIR458, a streptomycin-resistant derivative of strain 13, chloramphenicol- and streptomycin-resistant transconjugants were observed at consistently high frequencies ranging from 9.0×10^{-2} to 1.5×10^{-3} transconjugants/donor cell. A transconjugant from this mating, JIR4475, was also capable of transferring chloramphenicol resistance in subsequent matings performed by using JIR325 as the recipient and by selecting transconjugants on medium containing chloramphenicol, rifampin, and nalidixic acid. When a JIR325-derived transconjugant from this JIR4475 × JIR325 mating, JIR4479, was used as a donor, the frequency of transfer of chloramphenicol resistance to JIR458 was essentially the same as that observed in the original mating. Matings were also

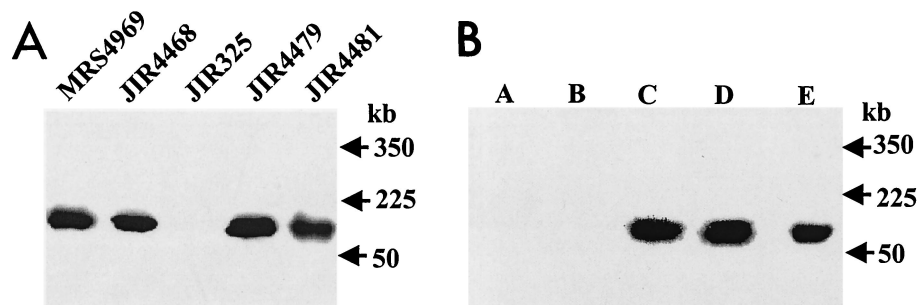


FIG. 3. PFGE/Southern hybridization analysis of undigested genomic DNA from parent strains and transconjugants. (A) Total undigested DNA from the strains indicated was separated by pulsed-field gel electrophoresis as previously described (5), with a Bio-Rad CHEF-DR II apparatus, where pulse times were ramped from 50 to 90 s over a period of 22 h. The gel was Southern blotted and probed with a 1.6-kb digoxigenin-labeled *cpe*-specific probe. Molecular sizes of the DNA markers are shown on the right. (B) Total undigested DNA from transconjugant JIR4479 was separated by pulsed-field gel electrophoresis, and the gels were Southern blotted and separately probed with the five digoxigenin-labeled *Cla*I fragments (lanes A through E) originally derived from pCW3 (see Fig. 4). For reference, the molecular sizes of the λ DNA standards (Bio-Rad) are shown on the right.

performed with other genetically marked *C. perfringens* recipients, JIR100, JIR39, and CW504, which have different genetic backgrounds. High-frequency transfer of chloramphenicol resistance was again observed (data not shown). Note that in each of the mating experiments, no colonies were observed on selection plates that contained only the donor or recipient strains. The presence of *cpe* Ω *catP* gene region in the resultant transconjugants was confirmed by PCR analysis using *cpe* primers located on either side of the *catP* gene (Fig. 1). The recipient strains were negative in this PCR whereas the transconjugants had a PCR band of the same size as that from the donor strain.

The mechanism of transfer of chloramphenicol resistance appeared to involve a conjugation-like mechanism since cell-to-cell contact between the donor and recipient was required. No chloramphenicol- and streptomycin-resistant colonies were obtained when culture supernatant from the donor was mixed with JIR325 cells or when a 0.45- μ m-pore-size Millipore membrane was placed between the donor and recipient strains. Furthermore, carrying out the matings in the presence of DNase I (1 mg/plate) had no effect on the frequency of transfer of the chloramphenicol resistance determinant.

Evidence that the transconjugants carry pMRS4969. Purification of large plasmids from *C. perfringens* cultures is a difficult task. Therefore, total genomic DNA was prepared from strains MRS4969, JIR325, JIR458, and JIR4468 and two additional independently derived transconjugants, JIR4475 and JIR4481. After digestion with *Hpa*I, Southern blots were hybridized with a *cpe*-specific probe (Fig. 2). No detectable hybridization signal was observed with *Hpa*I-digested DNA prepared from the *cpe*-negative recipient strains JIR325 and JIR458; however, as expected from previous observations (24), two hybridizing bands (3.0 and 8.5 kb) were observed with *Hpa*I-digested DNA from the original parent strain MRS4969. An identical hybridization pattern was seen with the pMRS4969 transformant, JIR4468, which was subsequently used as a donor in the matings, as well as the transconjugants JIR4479 and JIR4481 (Fig. 2). These results were as predicted for transconjugant strains derived from the conjugative transfer of pMRS4969.

To confirm these results and to determine whether the intact pMRS4969 plasmid had been transferred, pulsed-field gel electrophoresis (PFGE) was carried out with these strains, and the

resultant gel was Southern blotted and hybridized with the *cpe*-specific probe. In the absence of digestion with restriction endonucleases, only plasmid DNA molecules are able to enter the gel. The results showed that a single plasmid DNA band was detected in MRS4969 (Fig. 3). A hybridizing band of the same size was present in the transconjugants JIR4479 and JIR4481 but not in the recipient strains JIR325 (Fig. 3) and JIR458 (data not shown), providing clear evidence that pMRS4969 has been transferred intact from the donor strains to the recipients in these experiments.

The *cpe* plasmid pMRS4969 hybridizes to pCW3. Further hybridization studies were carried out to see if pMRS4969 had any regions of sequence similarity to pCW3 (1, 23), the conjugative tetracycline resistance plasmid that is homologous to all known conjugative *C. perfringens* plasmids (1–3, 16). Southern blots of *Bam*HI-digested total DNA preparations from JIR325 and JIR4468 were hybridized separately with digoxigenin-labeled probes prepared from separate recombinant plasmids (pJIR15 to pJIR18 and pJIR32), each of which carries one of the five *Cla*I fragments present in pCW3 (1). None of the five *Cla*I-derived probes hybridized with DNA from JIR325. However, all of the probes except pJIR17 hybridized to a >20-kb *Bam*HI fragment from JIR4468, with the hybridization of pJIR15 being noticeably weaker (Fig. 4).

Hybridization analysis of PFGE blots was used to confirm these results. In these experiments, the pJIR16, pJIR18, and pJIR32 probes hybridized to pMRS4969, but no hybridization was observed with pJIR15 or pJIR17 (Fig. 3).

The *cpe*⁺ parent plasmid of pMRS4969 represents the first conjugative virulence plasmid to be identified from *C. perfringens*. All other known conjugative plasmids from *C. perfringens* confer tetracycline resistance and have substantial similarity to pCW3 (2, 3). Although pMRS4969 does not confer tetracycline resistance, it still has significant similarity to pCW3. The two pCW3-derived *Cla*I fragments that have little or no similarity to pMRS4969 encode the tetracycline resistance determinant, which is found on pJIR17 (1), and the replication region, which is located on pJIR15 (P. Johanesen, K. Koutsis, D. Lyras, and J. I. Rood, unpublished data). It is tempting to speculate that the regions common to pMRS4969 and pCW3 are involved in conjugation, implying that these plasmids share

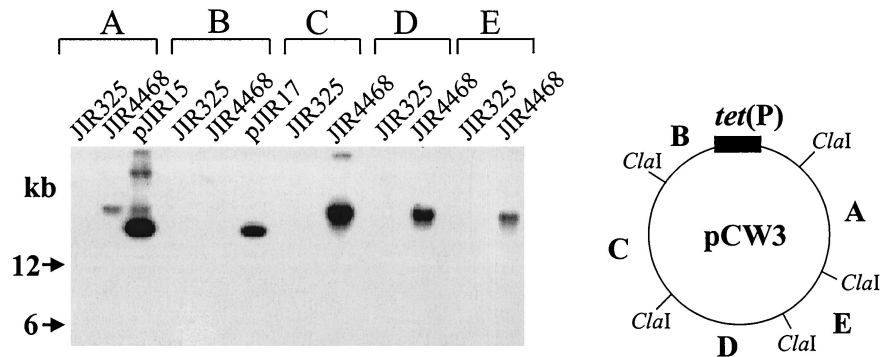


FIG. 4. Hybridization to pCW3-derived probes. Total DNA isolated from strains JIR325 and JIR4468 was digested with *Bam*HI, Southern blotted, and probed separately with the five pCW3-derived *Cla*I fragments that are present in the recombinant plasmids (1) pJIR15 (A), pJIR17 (B), pJIR16 (C), pJIR18 (D), and pJIR32 (E), as indicated. The pCW3 circular map shows the location of the five *Cla*I fragments (A through E).

a common conjugation mechanism. However, detailed DNA sequence analysis and genetic studies of both pCW3 and pMRS4969 need to be carried out before this hypothesis can be confirmed.

Implications for CPE-mediated diseases. Recent studies (25) suggest that the specific association between chromosomal *cpe* isolates and *C. perfringens* type A food poisoning is attributable, at least in part, to the chromosomal *cpe* isolates being considerably more heat resistant than plasmid *cpe* isolates, which favors their survival in the cooked foods that constitute the primary vehicle for *C. perfringens* type A food poisoning (19). The basis for the strong association between plasmid *cpe* isolates and CPE-associated non-food-borne human GI diseases has remained unclear. However, by demonstrating that the *cpe* plasmid can be conjugatively transferred between *C. perfringens* isolates, our present investigation offers a potential explanation for the relationship between isolates that carry the *cpe* plasmid and CPE-associated non-food-borne human GI diseases. That is, in vivo conjugative transfer of the *cpe* plasmid to normal intestinal flora isolates of *C. perfringens* may be important for establishing CPE-associated non-food-borne GI infections. Unlike *C. perfringens* type A food poisoning, which occurs upon ingestion of food containing massive numbers of *C. perfringens* cells that have a chromosomal *cpe* gene (19), CPE-associated non-food-borne human GI diseases appear to result from the ingestion or inhalation of low numbers of *cpe*-positive *C. perfringens* spores present in the environment (7). It is possible that the relatively few vegetative cells arising from the germination of these spores have the ability to transfer their *cpe* plasmid by conjugation to the much more abundant, but naturally *cpe*-negative, normal flora strains of *C. perfringens* already present in the GI tract. This process would convert these normal flora isolates to strains capable of causing enteric disease. Since the normal flora are well-adapted for intestinal colonization and growth, this in vivo conversion would hasten disease onset and may even be required to reach the in vivo levels of *cpe*-positive *C. perfringens* cells necessary for initiating enteric disease.

In vivo conjugative transfer of the *cpe* plasmid might also help explain why the GI symptoms derived from the non-food-borne isolates typically are more severe and longer in duration than those of *C. perfringens* type A food poisoning (6). Since *C. perfringens* strains in normal flora are proficient in their ability to colonize and persist in the GI tract, conjugative acquisition

of the *cpe* plasmid by these strains may increase the duration of GI infection. One consequence might be more chronic exposure to CPE, which could explain the more severe symptoms associated with CPE-associated non-food-borne diseases relative to those observed during acute *C. perfringens* type A food poisoning. The latter syndrome is caused by exogenous strains that are not necessarily well adapted to long-term survival in the human GI tract.

S. Brynestad and M. R. Sarker have contributed equally to the work and are joint first authors.

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