Necrotic Enteritis-Derived *Clostridium perfringens* Strain with Three Closely Related Independently Conjugative Toxin and Antibiotic Resistance Plasmids

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ABSTRACT **The pathogenesis of avian necrotic enteritis involves NetB, a pore-forming toxin produced by virulent avian isolates of *Clostridium perfringens* type A. To determine the location and mobility of the *netB* structural gene, we examined a derivative of the tetracycline-resistant necrotic enteritis strain EHE-NE18, in which *netB* was insertionally inactivated by the chloramphenicol and thiamphenicol resistance gene *catP*. Both tetracycline and thiamphenicol resistance could be transferred either together or separately to a recipient strain in plate matings. The separate transconjugants could act as donors in subsequent matings, which demonstrated that the tetracycline resistance determinant and the *netB* gene were present on different conjugative elements. Large plasmids were isolated from the transconjugants and analyzed by high-throughput sequencing. Analysis of the resultant data indicated that there were actually three large conjugative plasmids present in the original strain, each with its own toxin or antibiotic resistance locus. Each plasmid contained a highly conserved 40-kb region that included plasmid replication and transfer regions that were closely related to the 47-kb conjugative tetracycline resistance plasmid pCW3 from *C. perfringens*. The plasmids were as follows: (i) a conjugative 49-kb tetracycline resistance plasmid that was very similar to pCW3, (ii) a conjugative 82-kb plasmid that contained the *netB* gene and other potential virulence genes, and (iii) a 70-kb plasmid that carried the *cpb2* gene, which encodes a different pore-forming toxin, beta2 toxin.**

IMPORTANCE **The anaerobic bacterium *Clostridium perfringens* can cause an avian gastrointestinal disease known as necrotic enteritis. Disease pathogenesis is not well understood, although the plasmid-encoded pore-forming toxin NetB, is an important virulence factor. In this work, we have shown that the plasmid that carries the *netB* gene is conjugative and has a 40-kb region that is very similar to replication and transfer regions found within each of the sequenced conjugative plasmids from *C. perfringens*. We also showed that this strain contained two additional large plasmids that were also conjugative and carried a similar 40-kb region. One of these plasmids encoded beta2 toxin, and the other encoded tetracycline resistance. To our knowledge, this is the first report of a bacterial strain that carries three closely related but different independently conjugative plasmids. These results have significant implications for our understanding of the transmission of virulence and antibiotic resistance genes in pathogenic bacteria.**

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The production of potent protein toxins enables various isolates of *Clostridium perfringens* to cause a wide range of enterotoxemic and histotoxic diseases in both humans and animals (1–4). These diseases include avian necrotic enteritis, which is characterized by necrotic lesions in the small intestine (5–7). This disease is economically important to the poultry industry: acute clinical disease leads to increased mortality of birds, and subclinical disease leads to decreased weight gain and subsequent loss of productivity.

The pathogenesis of necrotic enteritis involves the pore-forming toxin NetB (8–10). The presence of the *netB* gene is strongly associated with strains derived from chickens with necrotic enteritis (11–14), and it has been shown that *netB*-positive strains produce necrotic lesions in avian challenge models (10, 15). The key role of NetB in the disease process has been demonstrated through the fulfillment of molecular Koch's postulates. A genetically defined *netB* mutant has been shown to be avirulent in

a chicken disease model, with virulence being restored by complementation with the wild-type *netB* gene (8).

C. perfringens strains can be divided into five toxin types, A to E, based on the extracellular toxins that they produce (16). Although alpha toxin, the major toxin implicated in *C. perfringens*-mediated human gas gangrene, is chromosomally determined, all of the other typing toxins are encoded on large plasmids (1, 17). In addition, the *C. perfringens* enterotoxin (CPE) gene is plasmid determined in some isolates, particularly those from animal or non-food-borne human infections (18–20). Genetic studies have shown that at least one CPE plasmid (21) and two epsilon-toxin plasmids (22) are conjugative. These plasmids and other toxin plasmids from *C. perfringens* (22–29), which presumably are also conjugative, all carry the *tcp* locus. This locus consists of 11 genes, several of which have been shown to be essential for conjugative transfer of the closely related tetracycline resistance plasmid pCW3 from *C. perfringens* (29–32). NetB is encoded on a plasmid of ca. 80 to 90 kb in size, as shown by pulsed-field gel electrophoresis and Southern hybridization, and is located within a 42-kb locus that appears to be specific to necrotic enteritis strains of *C. perfringens* (33).

In this study, we have examined the genetic location of the *netB* gene within the Australian necrotic enteritis isolate EHE-NE18. Using a genetically tagged EHE-NE18 Δ*netB*::*catP* derivative, we have demonstrated that the 82-kb plasmid that encodes NetB is conjugative. Furthermore, using a combination of conjugation experiments and high-throughput sequence analysis, we have shown that this strain harbors three closely related self-transmissible plasmids, each carrying a nearly identical copy of the *tcp* locus within a 40-kb region of nucleotide sequence similarity.

RESULTS

The *netB* toxin gene from strain EHE-NE18 is transferable. In previous studies, we generated a derivative of the necrotic enteritis strain EHE-NE18 in which the *netB* gene was replaced by the chloramphenicol (and thiamphenicol) resistance gene *catP* (JIR12231) (see Table S1 in the supplemental material) (8). Conjugation experiments were carried out in *C. perfringens* using strain JIR12231 (EHE-NE18 Δ*netB*::*catP*) as the donor and the rifampin (Rif^r) and nalidixic acid (Nal^r)-resistant strain JIR325 as the recipient in mixed-plate matings. From six independent experiments, it was found that thiamphenicol-resistant transconjugants were obtained at various frequencies that ranged from 5.3×10^{-7} to 6.6×10^{-2} transconjugants per donor cell, which suggested that the *netB* gene was located on a transferable element. The reason for this variability is not known. PCR analysis of several independently derived thiamphenicol-resistant transconjugants confirmed that they carried the 1.4-kb fragment derived from the insertionally inactivated *netB* gene (Fig. 1A, lanes 4 and 5). As expected, the *catP* gene was amplified from the transconjugants but not from the wild-type isolate, EHE-NE18.

The conjugative plasmid pCW3 carries the *tetA*(P)-*tetB*(P) operon, which is responsible for the tetracycline resistance phenotype of many *C. perfringens* strains (29, 34–37). PCR analysis showed that strain EHE-NE18 and its derivative EHE-NE18Δ*net*::*catP* were resistant to tetracycline and carried the *tetA*(P) gene (Fig 1A, lanes 6 and 7). Subsequently, we showed that tetracycline resistance could be transferred by conjugation from EHE-NE18Δ*netB*::*catP* at a frequency of 3.4×10^{-6} to 9.8×10^{-4} transconjugants per donor cell. A 764-bp *tetA*(P)-specific PCR

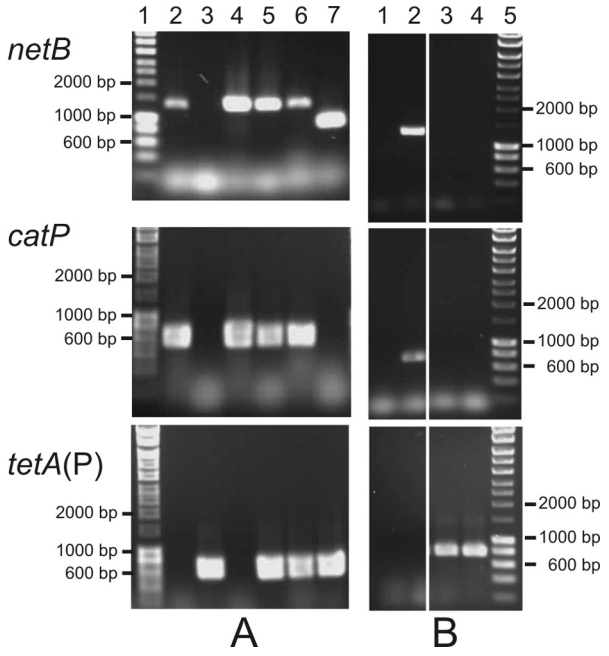

FIG 1 PCR analysis of transconjugants. DNA from the strains indicated was subjected to PCR analysis for the presence of the *netB*, *catP*, and *tetA*(P) genes, and the resultant products were separated by agarose gel electrophoresis. (A) PCR analysis of primary transconjugants. Lane 1, HyperLadder I (Bioline). The genomic DNA templates for the PCRs are as follows: lane 2, JIR12298 (JIR4394 transconjugant carrying the Δ*netB*::*cat*P plasmid pJIR3536); lane 3, JIR12295 (JIR325 transconjugant carrying the Tc^r plasmid pJIR3537); lane 4: JIR12293 (JIR325 transconjugant carrying pJIR3536); lane 5, JIR12290 (JIR325 transconjugant carrying pJIR3536 and the Tc^r plasmid pJIR3537); lane 6, JIR12231 (EHE-NE18 Δ*netB*::*cat*P carrying pJIR3536 and pJIR3537 and other plasmids); lane 7, EHE-NE18 (wild-type strain with the original *netB* plasmid pJIR3535 and pJIR3537). (B) PCR analysis of plasmid DNA isolated from the CW504-based secondary transconjugants. The templates are as follows: lane 1, CW504 (plasmid-free recipient); lane 2, JIR12308-derived pJIR3536 and pJIR3844, which was subsequently detected in this study; lane 3, pJIR3537 from JIR12309; lane 4, pCW3 from JIR4; lane 5, HyperLadder I (Bioline).

product could be amplified from all tetracycline-resistant transconjugants (Fig. 1A, lanes 3 and 5). We next examined whether the resistance determinants carried by strain EHE-NE18Δ*netB*::*catP* were always transferred together or transferred separately. In the primary matings from EHE-NE18Δ*net*::*catP*, we were able to obtain JIR325-derived Rif^r and Nal^r transconjugants that were resistant to thiamphenicol and susceptible to tetracycline (Tm^r Tc^s) (e.g., JIR12293 and JIR12294) or alternatively were tetracycline resistant and susceptible to thiamphenicol (Tc^r Tm^s) (e.g., JIR12295). The Tc^r Tm^s derivatives were isolated readily since 99% of the Tc^r transconjugants were Tm^s, whereas Tm^r Tc^s derivatives were obtained at a much lower frequency (10% of Tm^r transconjugants). Since these conjugation experiments were able to separate the thiamphenicol and tetracycline resistance determinants, we postulated that the *netB* gene and the *tetA*(P) genes were carried by two separate transferable genetic elements.

To confirm that the *netB* element was conjugative, we determined whether the Δ*netB*::*catP*-containing transconjugants could act as donors in subsequent transfer experiments. Since isogenic intrastrain mating experiments give higher and more consistent conjugation frequencies (38), the JIR325-derived transconjugants

TABLE 1 Conjugative transfer frequencies

Donor strain[a]	Donor plasmid	Phenotype[b]	Transconjugant		Resistance (%)[d]	
			Selection[b]	Transfer frequency[c]	Tc	Tm
JIR12293	pJIR3536	Tm^r	Tm Sm Chl	$(1.3 \pm 0.9) \times 10^{-2}$		
JIR12294	pJIR3536	Tm^r	Tm Sm Chl	$(3.9 \pm 0.3) \times 10^{-3}$		
JIR12295	pJIR3537	Tc^r	Tc Sm Chl	$(4.1 \pm 0.5) \times 10^{-2}$		
JIR12289	pJIR3536	Tm^r and Tc^r	Tm Sm Chl	$(1.2 \pm 0.6) \times 10^{-1}$	100	100
	pJIR3537	Tm^r and Tc^r	Tc Sm Chl	$(2.0 \pm 1.9) \times 10^{-1}$	100	30–50
JIR12290	pJIR3536	Tm^r and Tc^r	Tm Sm Chl	$(7.7 \pm 5.6) \times 10^{-2}$	100	100
	pJIR3537	Tm^r and Tc^r	Tc Sm Chl	$(4.1 \pm 5.0) \times 10^{-1}$	100	32–40

[a] Donors were the JIR325-based transconjugants indicated, and the recipient was the Sm^r Chl^r strain JIR4394.

[b] Tm, thiamphenicol; Tc, tetracycline; Sm, streptomycin; Chl, potassium chlorate.

[c] Number of transconjugants per donor cell.

[d] % resistance, Tc, refers to the number of transconjugants initially selected on thiamphenicol that when patched were found to be resistant to tetracycline; conversely, % resistance, Tm, refers to the number of transconjugants initially selected on tetracycline that were subsequently resistant to thiamphenicol.

were tested for their ability to act as donors in matings with strain JIR4394, a spontaneous streptomycin (Sm^r) and chlorate (Chl^r)-resistant mutant of strain 13, the parent of JIR325. The results showed that the thiamphenicol and tetracycline resistance markers could be transferred from separate Tm^r Tet^s (JIR12293 and JIR12294) and Tm^s Tet^r (JIR12295) donor strains at very high transfer frequencies (Table 1), which provided evidence that the tetracycline resistance determinant and the *netB* gene were located on independent conjugative elements. In addition, both the Δ*netB*::*catP* allele and the *tetA*(P) gene could be transferred from donor cells that contained both elements. When these Tm^r Tet^r transconjugants (JIR12289 and JIR12290) were used as donors, nearly all of the resultant second-generation Tm^r transconjugants were also Tc^r (Table 1). In contrast, only one-third of the Tc^r transconjugants were also Tm^r. This result implies that there was some variability in either the transferability or stability of these elements.

NetB is encoded on a conjugative plasmid. Since the Δ*netB*::*catP* and *tetA*(P) determinants were transferred independently at high frequencies, it was likely that these genes were located on separate conjugative plasmids. To examine this hypothesis and to characterize the resultant plasmids, we decided to determine their nucleotide sequences. The strain 13 derivatives that were used as the original conjugation recipients carry a plasmid, pCP13 (39), which would complicate the DNA sequencing of these elements. To simplify the plasmid purification and sequencing process, we transferred these putative plasmids by conjugation from the JIR4394-derived transconjugants (JIR12298 [Tm^r] and JIR12305 [Tc^r]) to the plasmid-free recipient strain CW504 (40), which generated strains JIR12308 (Tm^r) and JIR12309 (Tc^r), respectively. PCR analysis of purified total genomic DNA from these strains confirmed that the correct Δ*netB*::*catP*- and *tetA*(P)-specific products were detected in the respective Tm^r and Tc^r CW504-derived transconjugants. In addition, we used PCR primers specific for the pCP13-encoded *cna* gene to confirm that pCP13 had not been mobilized into the transconjugants.

The purification of large plasmids from *C. perfringens* is a very difficult task; nonetheless, large plasmids could be detected from plasmid DNA preparations purified from the CW504-derived transconjugants (Fig. 2A). These plasmids were designated pJIR3536 (carrying the Δ*netB*::*catP* allele) and pJIR3537 [which carried the *tetA*(P) gene]. PCR analysis (Fig. 1B) confirmed that pJIR3536 carried part of the *netB* gene and *catP*, as expected, and that pJIR3537 carried *tetA*(P). Restriction digests of these plasmids then were compared to equivalent digests of pCW3 (47 kb). The results (Fig. 2B) showed that tetracycline resistance plasmid pJIR3537 was just a little larger than pCW3, whereas the combined size of the DNA fragments present in the pJIR3536 digests was greater than 100 kb.

Southern hybridization analysis revealed that *catP*- and *tetA*(P)-specific probes hybridized to pJIR3536 and pJIR3537, respectively (Fig. 3), confirming that these genes were located on separate plasmids. In addition, a probe specific for *tcpF*, which is a conjugation-specific gene carried on pCW3 and other conjugative plasmids from *C. perfringens* (29), hybridized to both plasmids, which suggested that both the NetB toxin plasmid and the tetracycline resistance plasmid carried a Tcp conjugation locus. Analysis with a *plc*-specific probe, which hybridizes to *C. perfringens* chromosomal DNA, confirmed that these plasmid preparations did not contain detectable genomic DNA (data not shown).

Tetracycline resistance plasmid pJIR3537 is almost identical to pCW3. pJIR3537 DNA was purified from the CW504-derived transconjugant JIR12309 and sequenced using a Roche 454 GS-FLX sequencing system, producing 5 Mb of shotgun reads of an average length of 215 bp. These reads were assembled using the Newbler 2.0 software program (41), producing 17 contigs that included a 47-kb contig. PCR and conventional ABI 3730 capillary sequencing were used to finish the sequence and generate a circular plasmid of 48,787 bp, with an average depth of coverage of 65. The remaining contigs were low-coverage contigs from chromosomal contamination of the plasmid preparation, as determined by comparison with high-throughput sequencing reads derived from the recipient strain, CW504. The sequence of pJIR3537 was remarkably similar to that of the conjugative tetracycline resistance plasmid pCW3 (Fig. 4). It was 1,523 bp larger than pCW3, and the regions common to both plasmids were 99% identical at the DNA level. Both pJIR3537 and pCW3 had the same *tetA*(P) and *tetB*(P) tetracycline resistance genes and the same predicted plasmid replication and maintenance regions. All of the previously identified pCW3-derived *tcp* conjugation genes were present within pJIR3537, with the exception of the *tcpB* gene. The *tcpB* gene is also absent from several other pCW3-like plasmids and is not essential for conjugation (29, 31). Unlike pCW3, pJIR3537 contained a group II intron (C.p.I2) located in the intergenic region between *tcpG* and *tcpH*. This intron was found at the same location in the epsilon-toxin-encoding plasmid from the *C. per-*

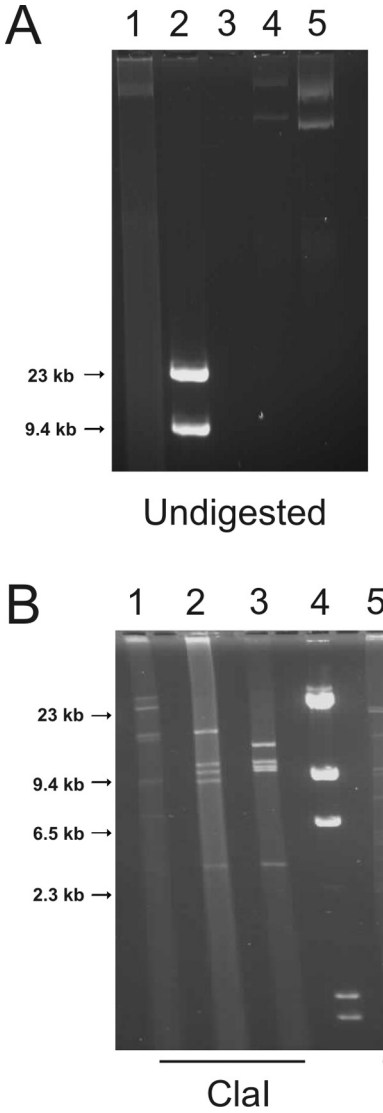

FIG 2 Analysis of *C. perfringens* plasmid DNA purified from CW504 derivatives. (A) Agarose gel electrophoresis of purified undigested plasmid DNA. Lane 1, plasmids from JIR12308, which includes pJIR3536 and pJIR3844; lane 2, HindIII-digested λ*cI857* DNA; lane 3, negative control, plasmid DNA preparation from the plasmid-free strain CW504; lane 4, pJIR3537 from JIR12309; lane 5, positive control, pCW3 from JIR4. (B) Agarose gel electrophoresis of purified plasmid DNA digested with the restriction endonucleases indicated. Lanes 1 and 5, JIR12308 (pJIR3536 and pJIR3844, which was detected subsequently in this study); lanes 2 and 6, JIR12309 (pJIR3537); lanes 3 and 7, JIR4 (pCW3); lane 4, HindIII-digested λ*cI857* DNA.

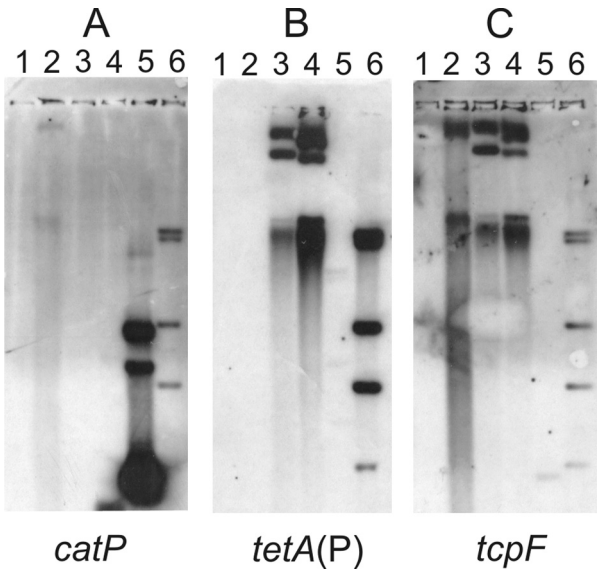

FIG 3 Southern hybridization analysis of purified plasmid DNA. Undigested plasmid DNA was separated by agarose gel electrophoresis, transferred to nitrocellulose, and blotted with the probes indicated. Lane 1, CW504 (plasmid-free negative control); lane 2, pJIR3536; lane 3, pJIR3537; lane 4, pCW3; lane 5, pJIR418 (*catP* positive control); lane 6, DIG-labeled HindIII-digested λ*cI857* DNA markers.

fringens type D strain JGS1721 (29). The plasmid pJIR3537 does not appear to encode any genes unique to necrotic enteritis strains and is therefore unlikely to be required for virulence.

Strain EHE-NE18 has three very similar large, independently conjugative, plasmids. The purified Δ*netB*::*catP* plasmid pJIR3536 from the CW504 derivative JIR12308 was sequenced using a Roche 454 GS FLX instrument, generating 12 Mb of shotgun reads with an average length of 232 bp. Sequencing data that corresponded to chromosomal DNA from CW504 were removed. Unlike the pJIR3537 data, the pJIR3536 reads did not assemble into a single near-complete contig. Newbler assembly of the data generated a large contig of 13,239 bp that contained the *netB*::*catP* gene region, but there was a very large number of reads that could not be satisfactorily assembled. Therefore, a second preparation of pJIR3536 plasmid DNA from the same strain was sequenced on an Illumina GAIIx instrument using 36-bp paired-end chemistry, yielding 413 Mb of reads. *De novo* assembly of these data using Velvet software (42) also failed to produce a satisfactory assembly. Repeated attempts to assemble these data yielded a variety of different contigs, none of which were larger than 13 kb, even though restriction analysis of pJIR3536 suggested that it was approximately 100 kb in size.

To solve this puzzle, a de Bruijn graph-based approach was used to represent and visualize the sequence reads (43). A de Bruijn graph of order k is constructed by breaking all reads into their overlapping subsequences of length k (called k-mers); each unique k-mer is a vertex in the graph, and an edge is drawn between two vertices if they overlap by $k - 1$ bp. A full and correct assembly is an unbroken path, or paths, through such a graph, passing through every part of the graph. If there are repeats, it may be necessary to pass through a section of the graph several times. For the pJIR3636 Illumina data set, a de Bruijn graph of order $k = 30$ was constructed from the 36-bp paired-end reads (5,417,911 pairs). To reduce the influence of sequencing errors, 30-mers occurring less than 10 times were discarded. As a final cleanup step, short "hairs" in this graph were removed, as were small fragments not connected to the main section of the graph. After cleaning, the graph consisted of 127,164 30-mers, with an average 30-mer depth of 147. A two-dimensional layout of this graph was constructed to allow visual examination of its structure (Fig. 5). In this layout, the Euclidean distance between each pair of nodes was made as close as possible to the length of the shortest path between them in the graph, as previously described (44). To aid in finding the correct path(s), indexes from k-mers to read pairs and vice versa were

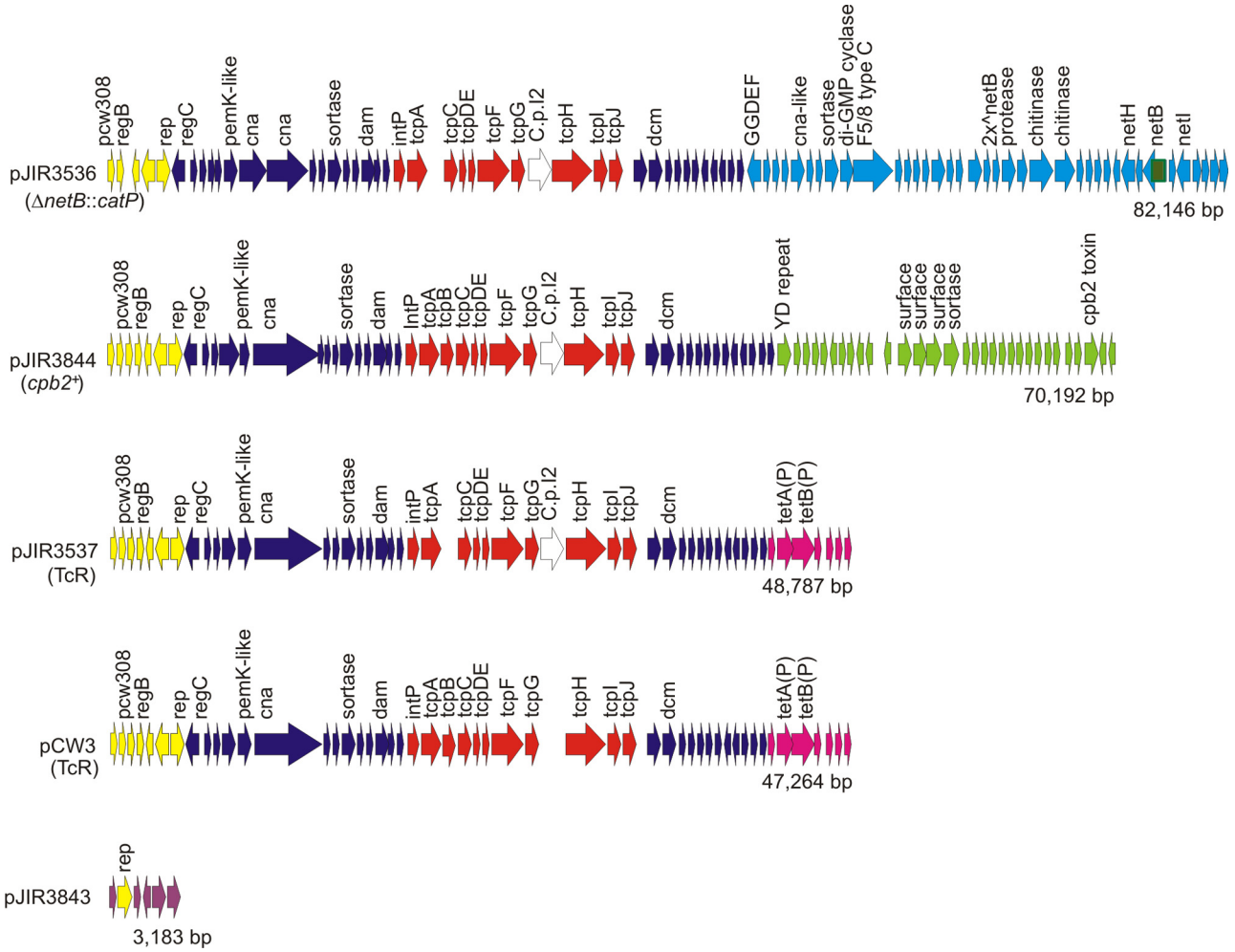

FIG 4 Comparative genetic maps of plasmids from the Δ*netB*::*catP* derivative of *C. perfringens* strain EHE-NE18. The four plasmids pJIR3536, pJIR3844, pJIR3537, and pJIR3843 are shown, and pCW3 is included for comparison. Open reading frames (ORFs) are represented by arrows. Yellow depicts genes that are likely to be involved in plasmid replication and maintenance, orange depicts genes from the *tcp* conjugation locus, dark blue depicts gene regions that encode proteins of unknown function but which are common to all known conjugative *C. perfringens* plasmids, light blue depicts genes present on pJIR3536, dark green depicts the *catP* insertion that replaces the majority of the *netB* gene, light green depicts genes present on pJIR3844, pink depicts genes that are present on pJIR3537 and pCW3, and purple depicts genes present on pJIR3843.

constructed from the Illumina reads, which had an insert size of ~200 bp, and also from the 454 data, with a larger insert size of ~3,000 bp (average length, 122 bp; 6,007 pairs). These indexes allowed for an interactive manual examination of pairing information on the graph layout and also further automated assembly of some sections of the graph.

Examination of the resultant de Bruijn graph (Fig 5) strongly suggested that the correct assembly would consist of two closed paths. Using a combination of automated and manual assembly, two closed paths through the graph were detected, and these paths were consistent with the read pairing information and between them covered the entire de Bruijn graph. The paths were identical along a large section (lower part of the layout) and then diverged to follow paths that were distinct, apart from some sections that were repeated several times in both paths (upper right of the layout), before gradually reconverging (upper left of the layout). Sanger sequencing was subsequently used to validate the data, confirm that these paths were correct, and resolve several small tandem repeat sections.

Based on this detailed analysis, we concluded that the pJIR3536 plasmid DNA preparation consisted of two independent plasmids that had 40 kb of very high nucleotide sequence identity. We were able to identify the circular plasmid molecule that corresponded to the Δ*netB*::*catP* plasmid pJIR3536. It was 82,146 bp in size and contained the complete 40-kb NetB pathogenicity locus, NELoc1, that was identified recently by others (33). pJIR3536 was also closely related to pCW3 (Fig. 4), the two plasmids having 40 kb of nearly identical sequence that included similar plasmid maintenance and replication regions, as well as the *tcp* conjugation locus. Like pJIR3537, pJIR3536 did not have the *tcpB* gene, and it had a group II intron inserted between *tcpG* and *tcpH*. Subsequent conjugation experiments led to the isolation of transconjugants that carried only pJIR3536. These plasmids were conjugative in subsequent matings, with high transfer frequencies, confirming that the *netB* plasmid encoded its own conjugative transfer, presumably by a *tcp*-mediated conjugation process. Further Illumina sequencing of DNA isolated from this strain confirmed our de Bruijn graph-mediated derivation of the sequence of this plasmid.

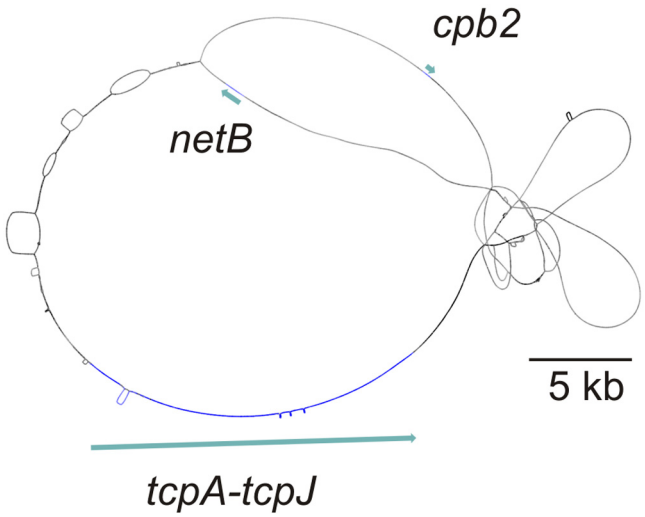

FIG 5 A de Bruijn graph resulting from the assembly of sequences derived from pIIR3536 and pJIR3844. Represented is a two-dimensional layout of the de Bruijn graph (43) formed by 127,164 30-mers obtained from Illumina sequencing of plasmid DNA from JIR12308, which carries pJIR3536 and pJIR3844. The *k*-mers have been arranged so that Euclidean distances in the diagram correspond as closely as possible to the shortest path through the graph. The pJIR3536 (*netB*) and pJIR3844 (*cbp2*) plasmids are paths through this diagram. The locations of the *netB*, *cpb2*, and *tcp* genes are indicated.

The second large plasmid detected by the de Bruijn graph analysis in the original pJIR3536 preparations was designated pJIR3844 and from our analysis was found to be 70,192 bp in size. It also had approximately 40 kb in common with pCW3, consisting of a similar maintenance and replication region and a *tcp* conjugation region that included a copy of the *tcpB* gene, as well as a group II intron (Fig. 4). Therefore, the original necrotic enteritis strain EHE-NE18 appears to carry three closely related plasmids that have essentially the same conjugation locus, a most unusual finding. pJIR3844 contained approximately 30 kb of sequence that was unique to this plasmid, including a beta2-toxin-encoding *cpb2* gene. The nucleotide sequence of this gene was distinct from that of the original *cpb2* gene isolated from the porcine *C. perfringens* type C strain CWC245 (45). It has been reported that there are two distinct families of *cpb2* genes within various *C. perfringens* isolates: the consensus and the atypical *cbp2* families (46). When the pJIR3844-derived *cpb2* gene was used to search the databases, it was found to be 95 to 100% identical to atypical *cpb2* genes from various nonporcine *C. perfringens* type A (JGS4147), type B (NCTC8533), type D (JGS4152) and type E (strain 853) strains. We subsequently used Targetron technology to insertionally inactivate the *cbp2* gene on pJIR3844 with an erythromycin resistance gene, generating two independently derived mutant plasmids, pJIR3967 and pJIR3969. Conjugation experiments showed that strains carrying these plasmids transferred their erythromycin resistance at a frequency of 1.7×10^{-2} transconjugants/donor cell and that this transfer occurred independently of the NetB or tetracycline resistance plasmids. These results confirmed that the *cpb2* plasmid encoded its own conjugative transfer, presumably due to its functional *tcp* locus. Therefore, we conclude that the three large plasmids present in the original necrotic enteritis strain EHE-NE18 are independently conjugative.

In whole-genomic DNA preps isolated from EHE-NE18Δ*net*::*catP*, a small ~3- to 5-kb plasmid was detected by gel electrophoresis. In the analysis of our high-throughput sequencing data, we consistently detected a small circularized contig that corresponded to the fourth plasmid in strain EHE-NE18. This small 3,183-bp plasmid, which we called pJIR3843, carried six putative genes (Fig. 4), including a gene that encodes a putative replication initiation protein with similarity to Rep proteins from small rolling-circle replication plasmids like pC194 from *Staphylococcus aureus*. Four of the remaining open reading frames encoded hypothetical proteins of unknown function, although the product of the final gene contained a phosphatidylethanolamine-binding domain (Pfam: PF1161.13).

Necrotic enteritis strain 56 carries similar NetB and beta2-toxin plasmids. Strain EHE-NE18 was isolated from an infected chicken in Australia. To investigate if similar plasmids were present in an avian necrotic enteritis strain from a different source, we examined derivatives of strain 56, which was isolated in Belgium (47, 48). Our analysis found that, like EHE-NE18, strain 56 was resistant to tetracycline and carried the *tetA*(P) gene. In addition, we found it had an atypical *cpb2* gene that had a sequence identical to that found in EHE-NE18. To further analyze the location of the *netB* gene within strain 56, we constructed a *netB* mutant of this strain by allelic exchange with a *catP* gene, in the same manner as previously described for strain EHE-NE18 (8). Conjugation experiments were performed using strain CW504 as a recipient, selecting for thiamphenicol resistance as before. The element carrying the Δ*netB*::*catP* gene was able to transfer at a frequency similar to that of pJIR3536 (4.2×10^{-4} to 6.3×10^{-2}). Most of the transconjugants were PCR positive for both Δ*netB*::*catP* and *cpb2*, but Δ*netB*::*catP* positive, *cpb2*-negative strains were also detected, at a lower frequency. High-throughput sequencing was performed with several strain 56 derivatives although the data could not be assembled into complete plasmids. Illumina sequence analysis of plasmid DNA isolated from a strain 56 Δ*netB*::*catP* transconjugant contained sequences that could be scaffolded to the entire sequence of pJIR3536. In contrast, sequences from the strain 56-derived *cbp2*-positive transconjugants did not contain all of the EHE-NE18-derived pJIR3844-like sequences. These data provided evidence that the Belgian strain 56 carried a conjugative *netB* plasmid that was closely related to the *netB* plasmid from the Australian isolate EHE-NE18 but suggested that the *cbp2* genes carried by these strains were located on more-divergent plasmids.

DISCUSSION

In this study, we have shown that the NetB toxin is encoded on a large conjugative plasmid in two necrotic enteritis strains of *C. perfringens*. This result is consistent with studies that have shown that many *C. perfringens* toxins are encoded on large plasmids (17, 23–28, 49). Of the four major toxins used to type *C. perfringens* isolates, only the alpha-toxin gene is located on the chromosome; the genes that encode the beta, epsilon, and iota toxins are all are located on large plasmids (17, 24, 25, 27). In addition, many of the genes encoding other *C. perfringens* toxins, such as beta2 toxin, CPE, and lambda toxin, have been found on large plasmids (17, 25, 45, 49, 50).

All three of the large plasmids identified in this study from strain EHE-NE18 carry the *tcp* locus, which has been found in each of the conjugative *C. perfringens* plasmids studied to date, including the well-studied tetracycline resistance plasmid pCW3 (29). This locus consists of 11 genes, *intP* and *tcpA* to *tcpJ*, and is re-

quired for conjugative transfer (29–32). In addition to the plasmids reported here, there are five fully sequenced large *C. perfringens* plasmids, which include pCW3, pCP8533etx, a plasmid that carries the *etx* and *cpb2* genes, pCP13, which carries *cpb2*, and the CPE plasmids pCPF4969 and pCPF5603, which have the *cpe* and *cpb2* genes (23, 26, 29, 39). Of these plasmids, only pCP13 does not have a *tcp* locus. Both pCW3 (tetracycline resistance) and pCPF4969 (CPE) have been shown to be conjugative (21, 23, 29), as have two epsilon-toxin plasmids (22) and a lincomycin resistance plasmid, pJIR2774 (51). All of these conjugative plasmids have the *tcp* locus. Therefore, in the absence of any other recognizable transfer region, we postulate that all three conjugative plasmids identified in this study are transferred by the same Tcp-mediated conjugation process.

The necrotic enteritis strain EHE-NE18 is resistant to tetracycline, and our conjugation studies confirmed that this resistance determinant is encoded on the conjugative plasmid pJIR3537. The sequence of pJIR3537 is remarkably similar to that of pCW3, having 99% nucleotide sequence identity. These two plasmids contain the same plasmid replication genes, a nearly identical *tcp* locus, and the same *tet*(P) tetracycline resistance determinants. Not only were the inducible tetracycline resistance genes nearly identical, but so were the two accessory genes present immediately downstream of the *tet*(P) determinant. These genes may be involved in regulating the expression of the tetracycline resistance operon (37, 52).

We have determined the complete nucleotide sequence of the conjugative Δ*netB*::*catP* plasmid pJIR3536 and by inference the sequence of its *netB*+ parent plasmid, designated pJIR3535, from the Australian necrotic enteritis strain EHE-NE18. In addition, we also have shown that in the Belgian necrotic enteritis strain 56 NetB toxin is encoded by a conjugative plasmid (pJIR3845) that contains all of the sequences present in pJIR3535. Based on these results, we postulate that the *netB* gene is likely to be found on similar conjugative plasmids in other *C. perfringens* strains isolated from cases of necrotic enteritis. These results are in very good agreement with the findings of a recent study (33) that showed that in several necrotic enteritis-derived *C. perfringens* strains, the *netB* gene is present as part of a 42-kb pathogenicity locus designated NELoc1 and was located on a ~80- to 90-kb plasmid, as determined by pulsed-field gel electrophoresis. Our analysis showed that the complete NELoc1 locus was present within the conjugative EHE-NE18 *netB* plasmid pJIR3535. The remainder of the plasmid comprised the backbone that was common to each of the conjugative *C. perfringens* plasmids that have been sequenced and included the predicted plasmid maintenance genes, the conjugation-related *tcp* locus, and other common regions. Therefore, the *netB* plasmid appears to consist of a pCW3-like plasmid in which the tetracycline resistance determinant has been replaced with the NELoc1 locus.

Strain EHE-NE18 has a third closely related plasmid, pJIR3844, which carries a *cpb2* gene that encodes beta2 toxin. We have insertionally inactivated the *cpb2* gene on this plasmid and have shown that the resultant genetically marked plasmid is also conjugative, which is consistent with the fact that it also carries the *tcp* locus. Beta2 toxin was originally detected in a *C. perfringens* strain of porcine origin (45). Two different forms, the consensus and atypical forms of beta2 toxin (60 to 70% amino acid sequence identity), have been observed (46, 53), with the consensus form most commonly linked to porcine *C. perfringens* strains from gastrointestinal infections (54). The *cpb2* gene carried by strain EHE-NE18 is identical to the atypical version. Another recent study found that two *C. perfringens* isolates from diseased chickens also carried the atypical *cpb2* gene (55). Preliminary analysis suggests that many *netB*-positive *C. perfringens* strains have the atypical *cpb2* gene, including the Belgian strain 56. However, not all of the genes found on the *cpb2* plasmid pJIR3844 were present within the strain 56 genome sequence. This result suggests that there may be less conservation among potential *cpb2* plasmids than among the NetB or tetracycline resistance plasmids. Further studies are required to determine the level of variation within the *netB* and *cpb2* plasmid families.

One of the surprising features of this study was the observation that one *C. perfringens* strain contained three closely related independently conjugative plasmids that had more than 40 kb of nearly identical sequence. These plasmids all carried essentially the same conjugation locus and therefore appeared to move by the same Tcp-mediated conjugation process. Previous studies have demonstrated that other *C. perfringens* strains from various sources can carry multiple plasmids and that some of these plasmids may carry the Tcp conjugation locus. However, it has not yet been shown experimentally that these plasmids are conjugative (24, 25, 27, 28). In this study, evidence that the *netB*, tetracycline resistance, and *cbp2* plasmids could each encode their own conjugative transfer was obtained by isolating separate transconjugants that each contained only a single plasmid and demonstrating that these single plasmids were still capable of conjugative transfer. To our knowledge this is the first time that such an observation has been made for any bacterial species. It will be interesting to determine how these plasmids are maintained independently in the one necrotic enteritis strain of *C. perfringens*. Preliminary bioinformatics analysis suggests that sequence differences in a ParM ortholog encoded in the replication and maintenance regions of these plasmids may be involved in this process. Studies are currently under way to confirm this hypothesis.

It is easy to envisage how homologous recombination between similar plasmid regions could lead to the evolution of a single conjugative plasmid encoding all of the required phenotypic functions. However, such a plasmid has not been observed. Perhaps the need for the host bacterium to retain the ability to separately respond to the presence of tetracycline or to environmental conditions conducive to necrotic enteritis has maintained an independent selection for the tetracycline resistance and NetB plasmids. The reason for the maintenance of what appears to be a more-variable *cbp2* plasmid is less clear, but it is unlikely to be related to necrotic enteritis infections since there is no evidence that beta2 toxin is involved in this disease. Further studies are under way to determine whether, as suspected, the presence of closely related, functional conjugative Tcp plasmids is a common phenomenon in non-necrotic enteritis strains of *C. perfringens*.

MATERIALS AND METHODS

Bacterial strains and culture conditions. All *C. perfringens* strains used in this study are listed in Table S1 in the supplemental material. These strains were grown in FTG broth (Oxoid) and nutrient agar (38) containing glucose (0.375% [wt/vol]). For matings, brain heart infusion (BHI) agar (Difco) was used. When required, solid media were supplemented with antibiotics at the following concentrations: thiamphenicol (Tm), 10 μg ml−1; tetracycline (Tc), 10 μg ml−1; rifampin (Rif), 20 μg ml−1; nalidixic acid

(Nal), 20 μg ml−1; streptomycin (Sm), 1 mg ml−1; and saturated potassium chlorate (Chl), 1% (vol/vol). Agar cultures were incubated overnight at 37°C in an anaerobic jar containing 10% CO_2, 10% H_2, and 80% N_2.

Conjugation experiments. All matings were carried out as described previously (38, 56). The transconjugants were selected on nutrient agar supplemented with the required antibiotics, and the conjugative transfer frequency was expressed as the number of transconjugants per donor cell.

Molecular analysis. Plasmid DNA was isolated from CW504-derived transconjugants by use of a Qiagen large-construct kit as described by the manufacturer, except that 600 ml of TPYG broth (57) was used for each *C. perfringens* strain. Genomic DNA was extracted from *C. perfringens* cells grown overnight in FTG broth, using the method previously reported for *Clostridium difficile* (58). The DNA concentration was measured by using a NanoDrop spectrophotometer (NanoDrop Technologies). Plasmid DNA was subjected to electrophoresis on an 0.8% agarose gel overnight at 35 V and visualized by a bioimaging system (Syngene) after ethidium bromide staining of the gel. PCR amplification used Taq DNA polymerase (Roche) and a 0.5 μM concentration of each primer (see Table S2). Denaturation (94°C for 30 s), annealing (55°C for 30 s), and extension (72°C for 1 min) steps were performed for 35 cycles. For Southern hybridization experiments, PCR products were purified using the QIAquick PCR purification kit (Qiagen) before digoxigenin (DIG) labeling. DIG labeling of probes, hybridization, chemiluminescent detection, stripping, and reprobing were all performed as described in the DIG user's manual (Roche).

Sequencing and analysis. High-throughput sequencing data were generated using a Roche 454 GS-FLX system and an Illumina GAIIx instrument. The resultant data were *de novo* assembled using Newbler 2.0 (41) or Velvet (42), respectively. Assemblies that were not resolved using these programs were analyzed using a de Bruijn graph approach (43). PCR amplification and subsequent Sanger sequencing on pJIR3536 and pJIR3844 were used to validate the assemblies.

Construction of *cbp2* mutant. *cpb2* mutagenesis was performed essentially as described for the *virR* gene using a derivative of the pJIR3566 targetron vector (59). To identify potential targetron insertion sites, the nucleotide sequence of *cpb2* from EHE-NE18 was submitted to the SigmaTargeTron design site (http://www.sigma-genosys.com/targetron/). Of the predicted sites, insertion into the sense strand at a position 390/391 bp from the ATG start codon in *cpb2* was selected for targetron modification. To retarget the group II intron, primer-mediated mutation by PCR was carried out with the IBS, EBS2, EBS1, and EBS universal primers (see Table S2 in the supplemental material) in accordance with the instructions from the TargeTron gene knockout system (Sigma-Aldrich). The 350-bp gel-extracted retargeting PCR product was digested with HindIII and BsrGI and ligated into similarly digested pJIR3566 DNA. The ligation mixture was used to transform *Escherichia coli* TOP10 cells (Invitrogen), and plasmids from a selection of the resultant transformants were isolated and sequenced. A vector, pJIR3857, that contained the desired altered nucleotides was chosen for subsequent mutagenesis studies. Introduction of the targetron plasmid, subsequent screening, and mutant confirmation were performed as previously reported (59).

Nucleotide sequence accession numbers. GenBank accession numbers are JN689219 (pJIR3536), JN689220 (pJIR3537), JN689218 (pJIR3843), and JN689217 (pJIR3844).

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SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at http://mbio.asm.org/lookup/suppl/doi:10.1128/mBio.00190-11/-/DCSupplemental.

Table S1, DOC file, 0.1 MB.

Table S2, DOC file, 0.1 MB.

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