Sequence of Two Plasmids from *Clostridium perfringens*
Chicken Necrotic Enteritis Isolates and Comparison with *C. perfringens* Conjugative Plasmids

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Abstract

Twenty-six isolates of *Clostridium perfringens* of different MLST types from chickens with necrotic enteritis (NE) (15 netB-positive) or from healthy chickens (6 netB-positive, 5 netB-negative) were found to contain 1–4 large plasmids, with most netB-positive isolates containing 3 large and variably sized plasmids which were more numerous and larger than plasmids in netB-negative isolates. NetB and cpb2 were found on different plasmids consistent with previous studies. The pathogenicity locus NELoc1, which includes netB, was largely conserved in these plasmids whereas NELoc3, present in the cpb2 containing plasmids, was less well conserved. A netB-positive and a cpb2-positive plasmid were likely to be conjugative, and the plasmids were completely sequenced. Both plasmids possessed the intact tcp conjugative region characteristic of *C. perfringens* conjugative plasmids. Comparative genomic analysis of nine CpCPs, including the two plasmids described here, showed extensive gene rearrangements including pathogenicity locus and accessory gene insertions around rather than within the backbone region. The pattern that emerges from this analysis is that the major toxin-containing regions of the variety of virulence-associated CpCPs are organized as complex pathogenicity loci. How these different but related CpCPs can co-exist in the same host has been an unanswered question. Analysis of the replication-partition region of these plasmids suggests that this region controls plasmid incompatibility, and that CpCPs can be grouped into at least four incompatibility groups.

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Introduction

*Clostridium perfringens* is an important pathogen of humans and animals, and certain strains of type A isolates cause necrotic enteritis (NE) in broiler chickens. NE is a common bacterial infection of chickens that is traditionally controlled by use of antibiotics. However the removal of growth-promoting antibiotics in broiler chickens in Europe and increasing demands elsewhere for “antibiotic-free” chicken are focusing efforts to find alternative approaches to control of this important disease [1,2]. There has been considerable effort in recent years to understand the pathogenesis of NE in chickens, including understanding the strains involved in disease.

A major breakthrough in understanding virulence in NE isolates of *C. perfringens* was the demonstration that a new toxin, NetB, was critical for development of the disease [3]. Subsequently, our group showed that netB formed part of a large pathogenicity locus (PAL) present on a plasmid (NELoc1) and that a second PAL, NELoc3, containing a gene named hbdA, which encodes 7-alpha-hydroxysteroid dehydrogenase, was also characteristic of NE isolates and was present on a separate plasmid [4]. A chromosomally encoded locus, NELoc2, was also present in all NE isolates. Pathogenicity loci are clusters of genes that harbour a group of potential virulence genes, which may contribute to the characteristic of the diversity of *C. perfringens* as a pathogen [5,6]. *Clostridium perfringens* produce at least 15 potent toxins that are responsible for severe diseases in humans and animals [7]. In *C. perfringens*, other than the mouse-lethal alpha toxin genes cpa and eto, which may sometimes be chromosomal, the major toxin genes (cpb, eto, usp) currently used to toxinotype *C. perfringens* strains as well as the netB, tcp2 and tcpL toxin genes are all harboured on large plasmids [4,7,8,9,10,11]. In general, NE strains carry 1 to 4 large plasmids which exhibit considerable diversity in size, ranging from ~35 kb to 100 kb [4]. These virulence plasmids share ~35 kb of conserved backbone sequence which contains among other genes the tcp conjugation locus belonging to a family of plasmids referred to as the pCW3-like family [12]. The tcp conjugation locus is present on all known conjugative plasmids from *C. perfringens* and consists of 11 genes (int P, tcpM to tcpF), of which tcpA, tcpF, tcpG, and tcpH are essential for conjugative transfer [12,13,14]. Conjugation systems are important contributors to the dissemination of antibiotic resistance determinants and virulence factors [15].
Recently, three plasmids from an NE isolate have been fully sequenced (pJIR3535, pJIR3844, pJIR3537); one of these contained netB and another the cpb2 gene; the third was a smaller tetracycline-resistance plasmid that did not contain virulence-associated genes [10].

In the study reported here, we sequenced two different plasmids containing NE-Loc1 and NE-Loc3 and analysed the diversity of plasmid profiles of a group of C. perfringens strains isolated from chickens. Further, comparative genomics tools were used to analyse DNA sequences. We found that both plasmids contained multiple genes which shared high similarity to well-known C. perfringens conjugative plasmids (CpCIPs).

Materials and Methods

Bacterial Strains and Media

Twenty six C. perfringens strains belonging to different multi-locus sequence types (ST) were examined (Table 1). NE strains, field isolates from NE cases, and healthy isolates from the same farm in Ontario as the outbreak flock, were obtained from Patrick Boerlin, Department of Pathobiology, University of Guelph [16]. Each isolate was grown overnight at 37°C under anaerobic conditions (80% N2, 10% H2, 10% CO2) on TGY medium (3% Tryptic Soy Broth [Difco Laboratories, Detriot, MI] containing 2% D-glucose [Difco], 1% yeast extract [Difco], and 0.1% L-cysteine [Sigma-Aldrich Co., St. Louis, MO]. All isolates were also cultivated in blood agar (Tryptic Soy Agar [Fisher] with 5% sheep blood) plates aerobically to confirm purity. E. coli strains were grown on Luria-Bertani agar plates (Difco) at 37°C in aerobic conditions.

Genomic and Plasmid DNA isolation

Genomic DNA was isolated from 3 ml of overnight culture in Brain Heart Infusion broth [Difco] at 37°C under anaerobic conditions [17]. After precipitation, DNA pellets were washed twice with 70% ethanol and resuspended in TE buffer (10 mM Tris-Cl, pH 7.5 1 mM EDTA). Plasmid DNA was purified using midi-Qiagen columns (Qiagen, Mississauga, Canada) following the manufacturer’s instructions.

Construction of netB and cpb2 Mutants

The generation of C. perfringens mutants was conducted as described in Heap et al. [18]. ClosTron intron targeting and design tool (http://clostron.com) identified possible intron target sites. The insertion sites in the sense strands at positions 461/462 bp in the netB open reading frame (orf) and 390/391 bp in the cpb2 orf were preferentially chosen to generate ClosTron-intron modifications, which were obtained by PCR from primers (IBS, EBS2, EBS1 and EBS universal) designed by the ClosTron website (Table S1). The 350-bp products and pMTL007 ClosTron-shuttle vector were both digested with HindIII and BglII, ligated and then transformed by heat shock into E. coli DH5α. Recombinant plasmids were isolated and sequenced in order to verify sequences of the retargeted intron specific for netB and cpb2 insertions. The recombinants pMTLnetB and pMTLcpb2 containing the modified netB and cpb2 intron were then electroporated into electrocompetent C. perfringens strain CP1. Recombinant insertions were selected and streaked onto BH1 agar containing 2.3 μg/ml of erythromycin to select for bacteria in which the intron had been inserted. Insertions were confirmed by PCR using the EBS universal primer and target gene specific reverse primers. ErnRAM-F and ErnRAM-R primers were used to demonstrate the ErnRAM splicing, primers used are shown in Table S1.

Conjugation Experiments

Plasmid transfer experiments were carried out with C. perfringens CW504 used as recipient strain. Overnight cultures of C. perfringens donor and recipient strains grown in TGY were mixed with a donor:recipient ratio of 2:1 and a total of 200 μl of both cultures were seeded onto BHI agar without antibiotics and incubated anaerobically at 37°C overnight. Subsequently, the bacterial growth was removed and resuspended in 3 ml of BHI broth. Transconjugants were selected on BHI agar plates supplemented with rifampicin (20 μg/ml), nalidixic acid (20 μg/ml) and erythromycin (2.5 μg/ml). Transconjugants were initially screened by PCR amplifications of specific genes (netB and cpb2) followed by Pulsed Field Gel Electrophoresis (PFGE) to analyze the presence of plasmids.

Pulsed Field Gel Electrophoresis

PFGE was performed to analyze the presence of plasmids in 26 poultry C. perfringens isolates, as described by Lepp et al. [4]. Briefly, DNA plugs for PFGE were prepared from overnight cultures of C. perfringens grown in TGY and the bacterial pellets incorporated into a final agarose concentration of 1% in PFGE certified agarose (Bio-Rad Laboratories, Hercules, CA). Plugs were incubated overnight with gentle shaking at 37°C in lysis buffer (0.5M EDTA pH 8.0, 2.5% of 20% sarkosyl (Sigma-Aldrich), 0.25% lysosome (Sigma-Aldrich) and subsequently incubated in 2% proteinase K (Roche Applied Science, Laval, QC) buffer for 2 days at 35°C. One-third of a plug per isolate was equilibrated in 200 μl of restriction buffer at room temperature for 20 min and then digested with 10 U of NotI (New England Biolabs, Pickering, ON) at 37°C overnight. Electrophoresis was performed in 1% PFGE-certified gel and separated with the CHEF-III PFGE system (Bio-Rad) in 0.56 Tris-borate-EDTA buffer at 14°C at 6 V for 19 h with a ramped pulse time of 1 to 12 s. Gels were stained in ethidium bromide and visualized by UV light. Mid-Range II PFGE markers (New England Biolabs) were used as molecular DNA ladder.

Preparation of DIG Probes and PFGE Southern Blotting

DNA probes for all PFGE Southern blot steps were labelled by PCR amplification in the presence of digoxigenin-11-UTP (DIG; Roche Applied Science) according to the manufacturer’s recommendation. DNA probes were amplified from C. perfringens strain CP1. DNA probes for netB and hdhA genes were prepared with specific primers (Table S1). DNA from PFGE gels was transferred to nylon membranes (Roche Applied Science, Mannheim, Germany). DNA hybridizations and detection were performed by using the DIG labelling and CSPD substrate according to the manufacturer’s recommendation (Roche). For Southern blot hybridizations, nylon membranes were prehybridized for at least 2 h at 42°C in hybridization solution without labelled probe and then hybridized separately at 42°C with specific DNA probes for 16 h. The membranes were washed at 68°C under high-stringency conditions. For each different DIG labelled probe, the membrane was first stripped with 0.2 N NaOH and 0.1% sodium dodecyl sulfate, incubated with prehybridization solution, and then reprobed.

Overlapping PCR Analysis of NE Locus 1–3

A battery of PCR reactions was performed to assess the conservation of NE-Loc1–3 among 11 selected poultry isolates. For NE-Loc1–3 reactions, a ready-to-use PCR mixture of Platinum PCR SuperMix high-fidelity kit (Invitrogen, Burlington, ON, Canada) was used in a 25 μl reaction containing 0.8 mM of
A touchdown PCR program was used: 94°C for 3 min, 35 cycles of 94°C for 15 s, 65°C to 50°C for 15 s/cycle (the annealing temperature is decreased by 1°C every cycle until 50°C), extension at 68°C for 5 min, and finally, 68°C for 10 min. For longer-range fragments the extension time was increased to 15 min. All primers used are described in Table S1. PCR product

| Table 1. General features of bacterial strains and plasmids. |
|---|
| **Strains/Plasmids** | **Sequence Type** | **Characteristics/Clinical signs** | **Source** |
| **E. coli** | | | |
| DH5α | F’ Φ80 lacZΔM15.1 (lacZYA-argF)U169 endA1 recA1 hsdR17(rk- mK- dK+) deoR thi-1 supE44 gyrA96 relA1 | Invitrogen |
| **C. perfringens** | | | |
| CW504 | Rif<sup>R</sup> Na<sup>R</sup> conjugation recipient | J.J. Rood, Monash University |
| CP1 | Necrotic enteritis | This study |
| CP1: netB::ErmRAM | Clostron insertion in netB gene | This study |
| CP1: cpb2::ErmRAM | Clostron insertion in cpb2 gene | This study |
| T98 | CW504 derived transconjugant Rif<sup>R</sup> Na<sup>R</sup> Erm<sup>R</sup> with plasmid pCpb2 from CP1: cpb2::ErmRAM | This study |
| **NE01** | 01 | Necrotic enteritis | [16] |
| **NE04** | 10 | Necrotic enteritis | [16] |
| **NE06** | 02 | Necrotic enteritis | [16] |
| **NE09** | 04 | Necrotic enteritis | [16] |
| **NE10** | 03 | Necrotic enteritis | [16] |
| **NE14** | 05 | Necrotic enteritis | [16] |
| **NE15** | 06 | Necrotic enteritis | [16] |
| **NE19** | 08 | Necrotic enteritis | [16] |
| **NE20** | 09 | Necrotic enteritis | [16] |
| **NE23** | 10 | Necrotic enteritis | [16] |
| **NE28** | 13 | Necrotic enteritis | [16] |
| **NE30** | 14 | Necrotic enteritis | [16] |
| **NE32** | 15 | Necrotic enteritis | [16] |
| **NE42** | 16 | Necrotic enteritis | [16] |
| **NE57** | 22 | Necrotic enteritis | [16] |
| **H<sup>+</sup>18** | 08 | Healthy | [16] |
| **H<sup>+</sup>22** | 01 | Healthy | [16] |
| **H<sup>+</sup>26** | 11 | Healthy | [16] |
| **H<sup>+</sup>27** | 12 | Healthy | [16] |
| **H<sup>+</sup>34** | 10 | Healthy | [16] |
| **H<sup>+</sup>60** | 06 | Healthy | [16] |
| **H<sup>-</sup>16** | 07 | Healthy | [16] |
| **H<sup>-</sup>45** | 17 | Healthy | [16] |
| **H<sup>-</sup>46** | 19 | Healthy | [16] |
| **H<sup>-</sup>47** | 18 | Healthy | [16] |
| **H<sup>-</sup>54** | 20 | Healthy | [16] |
| **Plasmids** | | | |
| pMTL007 | Clostridial vector for expression of Clostron, containing ErmRAM, ColE1, Cm<sup>R</sup> | [19] |
| pMTL-netB | pMTL007 containing intron retargeted to *C. perfringens* netB (sense insertion at 461–462 bp) | This study |
| pMTL-cpb2 | pMTL007 containing intron retargeted to *C. perfringens* cpb2 (sense insertion at 390–391 bp) | This study |

<sup>1</sup>Chalmers et al. 2008 [16].

<sup>2</sup>Rif<sup>R</sup>, Na<sup>R</sup>, Cm<sup>R</sup>, Erm<sup>R</sup>, refers to resistance to rifampicin, chloramphenicol and erythromycin, respectively.

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sizes were determined by agarose gel electrophoresis and visualized by ethidium bromide staining and photographed under UV light.

**Plasmids Sequencing and Sequence Assemblies**

The complete nucleotide sequence of two plasmids from *C. perfringens* pNetB and pCpb2 were determined using the 454 GS Junior Titanium platform (Roche Applied Science, Indianapolis, IN, USA). In brief, plasmid DNA (10 μg) was nebulized at 45 psi for 1 min to shear the DNA into fragments smaller than 400 bp. Sheared DNA was end repaired by incubating with 15 U of T4 polynucleotide kinase and 15 U of T4 DNA polymerase in the presence of buffer and a dNTP mix (10 mM each) at 12°C for 15 min and 25°C for 15 min. DNA was then purified by MinElute PCR Purification Kit (QiaGen). The 454-sequencing adapters were ligated to the DNA fragments according to the GS Junior Titanium shotgun DNA Library Preparation Method (Roche) by incubating with 104 U of ligase in the presence of ligase buffer at 15°C for 15 min and 25°C for 15 min. The nucleotide sequence reads obtained were assembled using the Newbler (version 2.5p1) de novo sequence assembly software (Roche). Gaps between contigs were closed by DNA amplification using conventional PCR techniques and Sanger sequencing.

**Sequencing and Annotation**

Complete sequences were automatically annotated by Rapid Annotation using Subsystem Technology (RAST) and manually rectified. BLASTN and BLASTX analyses were performed to compare the established sequences to known *C. perfringens* plasmids in the NCBI database.

**Comparative Analyses**

To analyse the similarity and the phylogeny of the *C. perfringens* conjugative plasmids (CpCps) the sequences of the sequenced plasmids pNetB-NE10 and pCpb2-CP1 were aligned with sequences of plasmids pCPF5603 (AB236337), pCPF4969 (NC_007772), pJIR3335 (JN689219), pJIR3844 (JN689217), pCPPB-1 (AB604032), pCPB533etx (NC_011412) and pCW3 (NC_010937) using the tool M-GCAT with default settings [19]. A custom PerlScript was used to visualize the alignment mapped on the respective GenBank files [20]. The computation of the core genome (predicted gene products encoded on every plasmid in this study) of the *C. perfringens* plasmids was carried out using the bioinformatics tool EDGAR [21]. The phylogenetic tree was built by the Neighbor-joining algorithm using MEGA5 software [22].

**Nucleotide Sequence Accession Numbers**

The CpCP sequences were assigned GenBank accession numbers JQ655731 and JQ655732 for pNetB-NE10 and pCpb2-CP1, respectively.

**Results**

**Pulsed-field Gel Electrophoresis and Southern Blot**

To determine the presence of large plasmids in NE and healthy chicken isolates, DNA from 26 poultry isolates (Table 1) were subjected to PFGE. In *silico* restriction endonuclease analysis of the genomes of *C. perfringens* strains SM101 and ATCC13124, as well as of plasmids pCPF5603, pCPF4969 and pCPB533etx, revealed that *NotI* cleaved the genomes at no more than one location, whereas all plasmids were cleaved once; this restriction enzyme was therefore chosen to linearize the plasmids prior to PFGE. The PFGE profiles of the virulent NE type A strains digested with *NotI* revealed the presence of one to four large plasmids ranging in size from 45 kb–150 kb in all strains (Figure 1, Table 2). PFGE analysis (Figure 1) showed the diversity of plasmids among the type A *C. perfringens* poultry isolates and their sometimes marked size variations, which were confirmed by PFGE/Southern blotting experiments (Figure 2). *C. perfringens* strains NE99 and NE10 carried just a single large netB-positive plasmid (Table 2) but most NE isolates carried at least 3 large plasmids, in which the *netB* and *cpb2-hdhA* genes were on distinct plasmids (Figure 2). Interestingly, a group of six *netB*-positive isolates from healthy chickens also showed three to four large plasmids, whereas five *netB*-negative healthy chicken isolates had fewer and smaller large plasmids (Figure S4A).

Southern blotting showed the presence of *cpb2* in two plasmids in the same isolate (Figure S4B). When *NotI*-digested genomic DNA was probed with *netB*, a hallmark of NELoc-1, it hybridized as a single large band in NE isolates as well as in *netB*-positive healthy chicken isolates (Figure S4C). Hybridization to ~80 kb to 100 kb bands confirmed the plasmid identity of these PFGE bands and showed that the *netB* gene was always located in the larger plasmids (Figure 2). The *hdhA* probe (NELoc-3) hybridized to different and smaller plasmids than the *netB*-probe (Table 2, Figure 2), in 15 of 15 virulent NE isolates as well as in all *netB*-positive healthy chicken isolates.

**Mutants and Conjugation**

The *netB* and *cpb2* genes were insertionally inactivated in the strain CP1, resulting in the mutant strains CP1ΔnetB::ErmRAM

**Figure 1. PFGE analyses of plasmids from NE *C. perfringens* poultry strains.** Agarose plugs containing DNA from each specified isolate were digested with *NotI* and subjected to PFGE and staining with ethidium bromide. Line numbers indicate isolate numbers M: Mid-Range II PFGE molecular DNA ladder (Kb). doi:10.1371/journal.pone.0049753.g001
and CP1\Delta\text{cpb2}::\text{ErmRAM}. The insertion of \text{ErmB}-carrying introns into the target genes was confirmed by PCR using primers flanking the insertion site (data not shown). The \text{netB} and \text{cpb2} genes located in different plasmids in CP1 strain were thus marked with erythromycin-cassette resistance (\text{ermB}) and this resistance could subsequently be used as a selective marker.

Conjugation assays were performed using \textit{C. perfringens} strains CW504 \text{Rif}\text{\textsuperscript{R}}\text{Na}\text{\textsuperscript{K}} as the recipient and CP1\Delta\text{cpb2}::\text{ErmRAM} and CP1\Delta\text{netB}::\text{ErmRAM} and CP1\Delta\text{netB}::\text{ErmRAM} as donor strains in plate matings. Both plasmids (pNetB and pCpb2) transferred to the recipient strain, however we were unable to find one transconjugant harbouring only pNetB. Erythromycin-resistant transconjugants were confirmed by specific PCR amplifications of \text{netB}::\text{ErmRAM} and \text{Acpb2}::\text{ErmRAM}; the \text{ermB} gene was amplified from the transconjugants but not from the wild-type or donor strains.

Conservation of NELoc-1, 2 and 3 in Poultry Isolates

Overlapping PCR assays were used to check the diversity of the three loci and their sites of insertion in nine virulent NE strains which represented different ST and plasmid profiles (classified by number and sizes) and two \text{netB}-positive isolates from healthy chickens (Figure S1). NELoc-1 showed a general uniformity and conservation. For NELoc-2 just one isolate (NE 30) showed no PCR amplification for reaction \#5 and two healthy chicken isolates (H26, H34) showed slightly smaller products. Most differences were found in the NELoc-3 (Figure S1).

Sequencing of Plasmids

The two plasmids pNetB-NE10 and pCpb2-CP1 were isolated from wild-type NE10 and transconjugant T98 (Table 1 and Figure S5) respectively and sequenced on the Roche 454 GS Junior system. The complete nucleotide sequences of the plasmids pNetB-NE10 and pCpb2-CP1 were assembled into circular DNA sequences of 81,693 bp and 65,875 bp with an average depth of coverage of 200, respectively (Figure 3). The average G+C content is 25.7\% for pNetB-NE10 and 26.8\% for pCpb2-CP1, which is very similar to the G+C content of most \textit{C. perfringens} plasmids [23].

\textbf{pNetB-NE10 and pCpb2-CP1}

Sequence annotation of pNetB-NE10 showed the presence of 82 open read frames (\text{orfs}) whereas pCpb2-CP1 contained 73 \text{orfs} (Figure 3). Both plasmids are organized in the typical plasmid backbone of other \textit{C. perfringens} plasmids [10,11,24]. Of the fully sequenced \text{QCPs}, the sequences of plasmids pNetB-NE10 and pCpb2-CP1 have identical gene organizations to plasmids pJIR3535 and pJIR3844 [10], respectively. The pNetB-NE10 and pCpb2-CP1 plasmids sequenced in this study are 99.1\% and 97.9\% similar to previous published plasmids pJIR3535 and pJIR3844, respectively. All these plasmids share a high degree of similarity with a major difference at the \text{orfs} 4 and 5 (Table S2).

\textbf{Comparative \textit{C. perfringens} Conjugative Plasmid Analyses}

The DNA sequences of plasmids pNetB-NE10 (JQ655731) and pCpb2-CP1 (JQ655732) were compared to those of plasmids pCPF5603 (AB236337), pCPF9469 (NC_007772), pJIR3535 (JN689219), pJIR3844 (JN689217), pCPPB-1 (AB604032), pCP8533ex (NC_011412) and pCW3 (NC_010937). Figure 4 shows a diagrammatic representation of the organization among these different \text{QCPs}. The software tool EDGAR [21] was used for the assessment of genes that are present on all nine \text{QCPs} and definition of a conserved backbone structure for these plasmids. A total of 24 core genes were identified (Table 3). From the core genes, 22 genes belong to the conserved backbone, encoding the
plasmid replication protein (rep), a DNA-binding transcriptional repressor (regD), the PemK protein, a sortase family protein, proteins required for conjugative transfer (tcpACDEFGHIJ), a DNA adenine-specific methyltransferase (dam), a tyrosine integrase (intP) and seven hypothetical proteins, for a total of around 35 kb of the plasmid.

A second comparative analysis that considered only plasmids from necrotic enteritis isolates showed that 39 common genes

Figure 3. Genetic maps of the sequenced NE plasmids pNetB-NE10 and pCpb2-CP1. The circles represent (from inner to outer most): (i) G + C skew; (ii) G + C content and (iii) open reading frames; arrows indicate the direction of transcription.
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Figure 4. Comparative analysis of C. perfringens conjugative plasmids. Comparative analysis of the sequenced NE plasmids pNetB-NE10 and pCpb2-CP1 and the published Cp plasmids pCPF5609, pCPF4969, pJR3844, pCPPB1, p8533etx and pCW3. Conserved regions within the analysed plasmids, pNetB (JQ655731), pCpb2 (JQ655732), pCPF5603 (A8236337), pCPF4969 (NC_007772), pJR3844 (JN689219), pJR3844 (JN689217), pCPPB-1 (A8604032), p8533etx (NC_011412) and pCW3 (NC_010937) are highlighted by grey boxes. Similarities between plasmids were calculated using the M-GCAT tool and visualised using PerlScript.
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among those plasmids (pNetB-NE10, pJR3535, pCpb2-CP1 and pJR3844) (Table 4) are conserved. These 39 genes additionally encode the LexA repressor (regB), replication protein (rep), DNA-binding transcriptional repressor (regD), PemK family protein, sortase protein, DNA adenine-specific methyltransferase (dam), tyrosine integrase (intP), conjugation proteins described above besides conjugation proteins TcpA and TcpC, group II intron reverse transcriptase LrLa, DNA-cytosine methyltransferase (dcm), and PemK, growth inhibitor (COG2337). All nine C. perfringens plasmids carry the rep gene, and encodes an ATPase involved in putative plasmid partitioning similar to the protein ParM of the ParMRC plasmid partitioning system.

**Phylogenetic Tree**

The sequences of the two sequenced NE plasmids pNetB-NE10 and pCpb2-CP1 and the seven completely sequenced CEs were analyzed phylogenetically (Figure 6) as described previously [18]. The phylogenetic tree suggests that these plasmids are closely related phylogenetically, and that there are closer relationships within each of the netB and the cph2-containing plasmids. Based on the homologous sequences of all plasmids the % identity varies between 92.3% (pCph2/pCPF4969) and 99.1% (pNetB/pJR3535).

**Discussion**

The current study provides complete DNA sequences of two NE C. perfringens virulence-associated plasmids (pNetB-NE10, pCpb2-CP1), further insight into the conjugative plasmids associated with NE, and significant new understanding of Clostridium perfringens conjugative plasmids.

In this study, PFGE analyses revealed the presence of one to four large plasmids >45 kb in fifteen NE isolates of known virulence and different MLST type [16]. The variation in size of

### Table 3. Core genome genes of C. perfringens plasmids.

| pNetB-NE10 | pCpb2-CP1 | Gene/orf | Name/Function |
|------------|-----------|----------|---------------|
| pNetB-NE10_1 | pCpb2-CP1_1 | hypothetical, unknown |
| pNetB-NE10_6 | pCpb2-CP1_6 | rep | plasmid replication protein |
| pNetB-NE10_8 | pCpb2-CP1_8 | regD | DNA-binding transcriptional repressor |
| pNetB-NE10_9 | pCpb2-CP1_9 | hypothetical, unknown |
| pNetB-NE10_10 | pCpb2-CP1_10 | hypothetical, unknown |
| pNetB-NE10_11 | pCpb2-CP1_11 | pemK | PemK, growth inhibitor (COG2337) |
| pNetB-NE10_14 | pCpb2-CP1_14 | hypothetical, unknown |
| pNetB-NE10_15 | pCpb2-CP1_15 | srt | sortase family protein |
| pNetB-NE10_16 | pCpb2-CP1_16 | hypothetical, unknown |
| pNetB-NE10_17 | pCpb2-CP1_17 | hypothetical, unknown |
| pNetB-NE10_18 | pCpb2-CP1_18 | dam | DNA adenine-specific methyltransferase |
| pNetB-NE10_19 | pCpb2-CP1_19 | hypothetical, unknown |
| pNetB-NE10_21 | pCpb2-CP1_21 | intP | tyrosine integrase |
| pNetB-NE10_22 | pCpb2-CP1_22 | tcpA | conjugation protein TcpA, FtsK/SpoIIE DNA translocase |
| pNetB-NE10_23 | pCpb2-CP1_23 | tcpC | conjugation protein TcpC |
| pNetB-NE10_24 | pCpb2-CP1_24 | tcpD | conjugation protein TcpD |
| pNetB-NE10_25 | pCpb2-CP1_25 | tcpE | conjugation protein TcpE |
| pNetB-NE10_26 | pCpb2-CP1_26 | tcpF | conjugation protein TcpF |
| pNetB-NE10_27 | pCpb2-CP1_27 | tcpG | conjugation protein TcpG |
| pNetB-NE10_29 | pCpb2-CP1_30 | tcpH | conjugation protein TcpH |
| pNetB-NE10_30 | pCpb2-CP1_31 | tcpI | conjugation protein TcpI |
| pNetB-NE10_31 | pCpb2-CP1_32 | tcpJ | conjugation protein TcpJ |
| pNetB-NE10_40 | pCpb2-CP1_33 | hypothetical, unknown |
| pNetB-NE10_42 | pCpb2-CP1_34 | hypothetical, unknown |

Core genome composed of 24 genes of the nine C. perfringens plasmids pCPF4969, pCPF5603, pJR3844, pCP8533ex, pCPPB-1, pCpb2-CP1, pNetB-NE10, pCW3 and pJR3535. The core genome was computed with the software tool Edgar. doi:10.1371/journal.pone.0049753.t003
the plasmids reported by us here as well as previously [4] suggests that numerous rearrangements occur between and within the large conjugative plasmids, although further plasmid characterization is required to confirm this. For example, Southern blotting showed that numerous rearrangements occur between and within the large plasmids reported by us here as well as previously [4] suggests that numerous rearrangements occur between and within the large conjugative plasmids, although further plasmid characterization is required to confirm this. For example, Southern blotting showed.

## Table 4. Core genome genes of NE *C. perfringens* plasmids.

| pNetB-NE10 | Gene/orf | Name/Function |
|------------|----------|--------------|
| pNetB-NE10_1 | hypothetical protein, unknown |
| pNetB-NE10_2 | regB | SOS-response repressor and protease LexA |
| pNetB-NE10_6 | rep | Plasmid replication protein |
| pNetB-NE10_8 | regD | DNA-binding transcriptional repressor |
| pNetB-NE10_9 | hypothetical protein |
| pNetB-NE10_10 | cytochrome-b-lac operon regulatory gene |
| pNetB-NE10_11 | pemK | PemK family protein |
| pNetB-NE10_13 | hypothetical protein |
| pNetB-NE10_14 | hypothetical protein |
| pNetB-NE10_15 | srt | Sortase protein |
| pNetB-NE10_17 | hypothetical protein |
| pNetB-NE10_18 | dam | DNA adenine-specific methyltransferase |
| pNetB-NE10_19 | hypothetical protein, unknown |
| pNetB-NE10_20 | hypothetical protein, unknown |
| pNetB-NE10_21 | intP | tyrosine integrase/recombinase |
| pNetB-NE10_22 | tcpA | FtsK/SpolII DNA translocase TcpA |
| pNetB-NE10_23 | tcpC | conjugation protein TcpC, putative Tn916 |
| pNetB-NE10_24 | tcpD | conjugation protein TcpD |
| pNetB-NE10_25 | tcpE | conjugation protein TcpE |
| pNetB-NE10_26 | tcpE | conjugation protein TcpE |
| pNetB-NE10_27 | tcpF | conjugation protein TcpF |
| pNetB-NE10_28 | G2 | group II intron reverse transcriptase LtrA |
| pNetB-NE10_29 | tcpH | conjugation pore, membrane protein TcpH |
| pNetB-NE10_30 | tcpI | conjugation protein TcpI |
| pNetB-NE10_31 | tcpJ | conjugation protein TcpJ |
| pNetB-NE10_33 | hypothetical protein, unknown |
| pNetB-NE10_34 | dcm | DNA-cytosine methyltransferase |
| pNetB-NE10_35 | hypothetical protein, unknown |
| pNetB-NE10_36 | hypothetical protein, unknown |
| pNetB-NE10_37 | hypothetical protein, unknown |
| pNetB-NE10_38 | conserved hypothetical protein, unknown |
| pNetB-NE10_40 | conserved hypothetical protein, unknown |
| pNetB-NE10_42 | hypothetical protein, unknown |
| pNetB-NE10_43 | nuclease family transposase |
| pNetB-NE10_52 | cell wall surface anchor family protein |
| pNetB-NE10_53 | srt | sortase A, LPXTG specific |
| pNetB-NE10_58 | Recombinase |
| pNetB-NE10_66 | hypothetical protein |

Core genome composed of 39 genes of the five *NE C. perfringens* plasmids type A, pJIR3844, pCpb2-CP1, pNetB-NE10, pJIR3535. The core genome was computed with the software tool Edgar.

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## Figure 5. Comparative analysis of central control region of *C. perfringens* conjugative plasmids.

Comparative genomic analysis of the central control region of *C. perfringens* plasmids starting from *regB* regulatory gene. Identical colors designate similar function on pNetB-NE10, pCpb2-CP1, pCPF5609, pCPF4969, pJIR3535, pJIR3844, pCPPB1, pCPS533etx and pCW3.

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The healthy *netB*-negative chicken isolates lacked the NEloc1 and NEloc3 and their related plasmids, supporting the role of these plasmids in NE.

Overlapping PCR of the three pathogenicity loci [4] confirmed that NEloc-1, which contains *netB*, is very conserved (Figure S1). Size variation of pNetB plasmids therefore must be the result of other changes in these plasmids. In contrast, the NEloc-3 showed greater variation (Figure S1). For example, the isolates NE28, NE30 and H26 possessed just a fragment of the NEloc-3 (*hdhA* gene) and isolate NE42 seemed to harbour only the 5’ and 3’ links of this locus. This suggests that NEloc3, the smallest of the loci associated with NE isolates [4] is less important for NE than the other two loci. The chromosomal NEloc-2 was intact in all except one (NE30) of the eleven strains tested, confirming that it is an important signature for NE isolates. Conjugation assays using the erythromycin resistance-marked NE *C. perfringens* CP1 strain (which contains four large plasmids) as donor and the strain CW504 as recipient resulted in transconjugants with a variable number of the large plasmids (Figure S5), and suggests that all these plasmids are conjugative. Our sequencing data showed that that both pNetB-NE10 and pCpb2-CP1 possess the tcp conjugation region, which has been found in all of the conjugative *C. perfringens* plasmids to date [10,12]. The pNetB-NE10 and pCpb2-CP1 plasmids sequenced in this study are 99.1% and 97.9% similar at the nucleotide level to previous published plasmids pJIR3535 and pJIR3844, respectively (Table S2). The presence of intact tcp-based conjugative regions suggests that pNetB-NE10 and pCpb2-CP1 plasmids are conjugative, supporting the recent work of others that showed that pJIR3535 and pJIR3844 plasmids to be conjugative [10].

Analysis of the two new genome sequences of plasmids pNetB-NE10 and pCpb2-CP1 isolated from NE isolates *C. perfringens* (Figure 3) showed the high similarity with two other recently sequenced avian necrotic enteritis *C. perfringens* plasmids pJIR3535 and pJIR3844 [10] and confirmed the extensive conservation of the common backbone among all *CP*CPs [10,11,24,25,26].

Comparative genomic analysis showed that *CP*CPs, including the two plasmids described here, showed greater gene rearrangements including pathogenicity locus and accessory gene insertions around rather than within the backbone region (Figure 4). The *CP*CPs have a mosaic organization in which transposons and
The common core genome for NE-isolate associated plasmids is a conserved backbone region highlighted in gray. Hence, differences between the plasmids are related to their pathogenicity locus (yellow), also including different toxin genes (pink) (Figure 4). The nuclease family transposase recognized as orf50 in the pCW3 plasmid seems to be the insertion site of the pathogenicity loci of these conjugative plasmids leading to different organizational types (Figure 4). Inter- and intra-strain rearrangements of QCPs are apparently responsible for the large size variation of conjugative plasmids from NE *C. perfringens* isolates (Table 2; Figure 1 and 2) possibly by duplications, insertions and deletions. Figure 4 shows a clear pattern of organization around the backbone region and in the pathogenicity loci of the QCPs, and of the development of these plasmids. The *dcm* region has previously been described as a possible hot spot for insertion of iota-toxin genes in plasmids of *C. perfringens* type E [9], which supports our suggestion of insertional “hot spots” between the regulation and partitioning genes and downstream of the *tcp* transfer region, where *dcm* is located. The pattern that emerges in the analysis shown in Figure 4 is that the major toxin-containing regions of the QCPs are organized as pathogenicity loci. This was first described for the NELoc1 and NELoc3 of the NE-associated plasmids [4], but might be a general feature of these virulence plasmids.

The comparative analysis of all nine *C. perfringens* plasmids showed a core genome of 24 genes, most of them belonging to the conserved backbone structure (Table 3) which includes the transfer of a clostridial plasmid (*tcp*) locus. The backbone region comprises a large portion of the conjugative plasmids [10,11,25,26,27], so that for both NE virulence plasmids, as well as for other characterized major virulence plasmids, a size around 35 kb seems to be optimized for efficient replication, conjugative transfer, plasmid maintenance and stability functions. Within the backbone region, there is what we designate the central control region (CCR) consisting of the replication (*reg*), regulatory genes (*reg*) and putative partition genes (*parM*).

The common core genome for NE-isolate associated plasmids is larger (39 genes, around 41 kb). Comparison of the four NE-isolate-associated *C. perfringens* plasmids (pNetB-NE10, pJIR3535, pCpb2-CP1 and pJIR3844) identified not only 35 genes in the backbone region but interestingly also four genes common in the pathogenicity loci. These genes encode a cell wall surface anchor family protein, the sortase A, a resolvase/recombinase and a hypothetical protein. It is clear that *C. perfringens* conjugative plasmids are closely related since they show remarkable homology [8,11,27].

Plasmid partition is classified by one of three types of *par* systems. Type I systems use ParA ATPase proteins with Walker-type folds and centromere-binding proteins called ParB; type II systems use actin-like ParM ATPases and centromere-binding proteins called ParR; and a recently described type III system uses a tubulin-like protein, TubZ [28]. The ParMRC operon is a well-known partition system for bacterial DNA segregation in low copy number plasmids [29]. These partition systems consist of three components: two genes *parM* and *parR* located side-by-side, with *parM* encoding a NTPase protein, and ParR, a specific centromere-binding protein, and a *cis*-acting centromere-like site *parC*, a small non-coding plasmid region with a series of 11 bp repeats. Interestingly, the equivalent to the ParR protein was not found in any of the QCPs, but there is a gene adjacent to *parM*, transcribed in the same orientation as *parM*, that encodes a conserved protein of unknown function (orf4 in pNetB-NE10). Sequence differences in the ParM ortholog encoded in the replication and maintenance regions of these plasmids may be involved in this process. The mechanism of segregation is presently unknown for *C. perfringens* conjugative plasmids, and no Par system was described in their DNA sequence.

Six genes were found to be unique within the backbone region for the NE plasmids. These include the collagen adhesion protein (orf12) and a hypothetical protein (orf40) and four genes located in the CCR (orf3, orf4, orf5 (*parM*), orf7 (*regCB*), using pNetB-NE10 as reference). These differences in the CCR transcriptional regulatory genes and segregation genes suggest that these differences may allow this family of plasmids to co-exist in their *C. perfringens* host (Figure 1) and ensure equal inheritance by daughter cells during cell division. There is apparently a limit to the types of large plasmids that a host may carry (Figure 1), which...
may be a function of the limited variation in the CCR. Gurjar et al [27] had earlier suggested that only certain toxin plasmid combinations could be stably maintained within a single C. perfringens cell. Earlier analysis suggested that differences in ParM orthology may be involved in this process [10]. It will be of interest to examine the CCR regions of other CpCPs found in NE strains with multiple plasmids, to determine how these relate to the postulated incompatibility system described here.

The mechanism of the partitioning system incompatibility in CpCPs proposed here is different from the well-known replication-mediated incompatibility [30]. Two different plasmids with the same partitioning system cannot coexist stably in the same host because of the competition between identical partitioning systems [31,32,33]. Based on this comparative genomic analysis, we suggest that C. perfringens conjugative plasmids can be grouped according to their types of putative partitioning genes, in particular parM (orf3) and orf4 (hypothetical protein), which seems to be equivalent to parR in the ParMRC system.

ATPase/ParM protein showed the highest similarity (99%) in amino acid sequence in the group of plasmids pNetB-NE10 (orf5), pJIR355 (orf0004); pCPF4969 (orf1), pCPF5603 (orf16) and pCPPB-1 (orf63) (Figure 5) (Fig S2). Amino acid sequence alignments showed that the ParM proteins contain conserved domain actin-like ATPases (PRK13917) and a predicted function of a plasmid segregation protein as part of a type II Par system [28,29]. Plasmids pCpb2-CP1 (orf5) and pJIR3844 (orf6) form a second group that encodes a different ATPase with no conserved domain and just 27% protein identity with orthologues of the first plasmid group. Although ATPase/ParM proteins from plasmids pCW3 (orf13) and pCP8533etx (orf52) have low homology (27%) with each other, both proteins belong to a superfamily of StbA proteins, a family that consists of several bacterial StbA plasmid stability proteins.

The orf4 gene in pNetB-NE10 and its homologues in other CpCPs have no conserved domain or significant similarity to other known proteins in GenBank. Speculatively, this hypothetical protein is suggested to be the potential ParR component of the partitioning system of CpCPs, primarily because it is located adjacent to parM with the same transcriptional orientation in all CpCPs analysed. Interestingly, this hypothetical protein is also conserved in the same plasmid groups described above, as is shown in the multiple sequence alignments (Figure S2). Another important element to complete the ParMRC system is the presence of parC, the centromeric region of the plasmid. Centromeres consist of a series of tandem DNA repeats of eight 10-bp or four 20-bp repeats typically located adjacent of the parM gene [28,29]. However, the precise size and organization of the parC site varies among ParMRC system [29]. The upstream sequence of parM genes of the nine CpCPs revealed several imperfect 11 bp repeats and conserved regions among the sequences which appear to be the equivalent of a parC site (Figure S3).

In conclusion, the complete sequencing of two new conjugative plasmids from NE isolates described here, when combined with comparative analysis of previously sequenced plasmids, adds considerably to understanding the evolution of virulence-associated plasmids in C. perfringens, and contributes to the unanswered question of how these different but related plasmids can co-exist in the same host. The suggestion proposed here of classifying CpCPs into incompatibility groups, of which four are described here, based on the partitioning systems, requires confirmation by experimental data. There are important areas still to be understood including the function of conserved hypothetical proteins, the presence of additional plasmid incompatibility systems, and the basis of any limitation of specific CpCP family members to particular C. perfringens types. Sequencing of further large CpCPs (Figure 1) might add confirmation to our supposition about the role of the CCR in maintenance of different family members in the same host.

Supporting Information

Figure S1 Overlapping PCR analysis of NE locus in C. perfringens. PCR reactions were performed using DNA from C. perfringens strains described on Table 1. Healthy and NE C. perfringens isolates H16, H34, NE04, NE09, NE10, NE14, NE20, NE23, NE28, NE30, NE42, respectively. Genetic organization of NE loci. (A) Overlapping PCR analysis of NE locus 1. (B) Overlapping PCR analysis of NE locus 2. (C) Overlapping PCR analysis of NE locus 3. PCR products spanning the entire locus are represented by black bars and the PCR results for each strain tested are given below as follows: +, PCR product was of expected size; −, no PCR product produced. Where the PCR product did not match the expected size, the actual size is given.

Figure S2 Amino acid alignments of proteins encoded by different C. perfringens plasmids. Plasmid names and their respectiveorf number (plasmid name orf#) are described for each protein. Identical residues (*), conservative amino acid substitutions (.), and semi-conservative amino acid substitutions (:) are shown below the aligned sequences. (MUSCLE. −3.7).

Figure S3 Repeats found on the upstream region of parM gene. Possible tandem repeats found on the upstream region of parM gene next to rep gene from C. perfringens plasmids using etandem (http://emboss.bioinformatics.nl/cgi-bin/emboss/etandem).

Figure S4 PFGE and Southern blot analyses of plasmids from healthy C. perfringens poultry strains. (A) PFGE analyses of plasmids from healthy C. perfringens poultry strains. Agarose plugs containing DNA from each specified isolate were digested with NotI and subjected to PFGE and staining with ethidium bromide. See Table1 and 2 for isolate features. Line numbers indicate isolate numbers M: Mid-Range II PFG molecular DNA ladder (Kb). (B) PFGE Southern blot of plasmids from healthy C. perfringens poultry strains. Southern blotting of PFGE (Figure S4A) was performed with only DIG-labelled probe for cpb2 gene. M: Mid-Range II PFG molecular DNA ladder (Kb). (C) PFGE Southern blot of plasmids from healthy C. perfringens poultry strains. Southern blotting of PFGE (Figure S4A) was performed with only DIG-labelled probe for netB gene. M: Mid-Range II PFG molecular DNA ladder (Kb).

Figure S5 PFGE analyses of plasmids from transconjugants C. perfringens strains. Agarose plugs containing DNA from each specified isolate were digested with NotI and subjected to PFGE and staining with ethidium bromide. Lines indicate: CW504 recipient strain (plasmid free); T98 (transconjugant carrying the plasmid pCpb2); T117 (transconjugant carrying three of CP1 plasmids); T118 (transconjugant carrying four of CP1 plasmids); T119 (transconjugant carrying two of CP1 plasmids); T125(transconjugant carrying two of CP1 plasmids); T128 (transconjugant carrying two of CP1 plasmids); CP1 donor strain (harbours four large plasmids); M: Mid-Range II PFG molecular DNA ladder (Kb).
Table S1  List of primers. (A) Primers used for PCR DIG labelling and mutation (B) Primers used for overlapping PCR reactions of the three Pathogenicity loci characteristic of necrotic enteritis C. perfringens isolates.

(DOCX)

Table S2  Comparison of NE C. perfringens plasmids. (A) Comparison of coding sequences pNetB-NE10 and gJIR3355 NE C. perfringens plasmids by means of BLASTn analyses. Open reading frames are labeled according to the annotation of plasmid pNetB-NE10 (B) Comparison of open reading frames pCph2-CP1 and gJIR3844 NE C. perfringens plasmids by means of BLASTn analyses. Open reading frames are labeled according to the annotation of plasmid pCph2-CP1.

(DOCX)

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Author Contributions
Conceived and designed the experiments: VRP JFP. Performed the experiments: VRP MC. Analyzed the data: VRP MC FE JB JFP. Wrote the paper: VRP JFP.