The Specificity of ParR Binding Determines the Incompatibility of Conjugative Plasmids in Clostridium perfringens

Thomas D. Watts, Daouda A. K. Traore, Sarah C. Atkinson, Carmen Lao, Natalie Caltabiano, Julian I. Rood, Vicki Adams

Abstract

Plasmids that encode the same replication machinery are generally unable to coexist in the same bacterial cell. However, Clostridium perfringens strains often carry multiple conjugative toxin or antibiotic resistance plasmids that are closely related and encode similar Rep proteins. In many bacteria, plasmid partitioning upon cell division involves a ParMRC system; in C. perfringens plasmids, there are approximately 10 different ParMRC families, with significant differences in amino acid sequences between each ParM family (15% to 54% identity). Since plasmids carrying genes belonging to the same ParMRC family are not observed in the same strain, these families appear to represent the basis for plasmid compatibility in C. perfringens. To understand this process, we examined the key recognition steps between ParR DNA-binding proteins and their parC binding sites. The ParR proteins bound to sequences within a parC site from the same ParMRC family but could not interact with a parC site from a different ParMRC family. These data provide evidence that compatibility of the conjugative toxin plasmids of C. perfringens is mediated by their parMRC-like partitioning systems. This process provides a selective advantage by enabling the host bacterium to maintain separate plasmids that encode toxins that are specific for different host targets.

Importance

Toxins produced by the Gram-positive pathogen Clostridium perfringens are primarily encoded by genes found on different conjugative plasmids. These plasmids encode highly similar replication proteins and therefore should be incompatible, but they are often found to coexist within the same isolate. In this study, we showed that a series of phylogenetically related ParMRC plasmid partitioning systems, structures that are normally responsible for ensuring that plasmids segregate correctly at cell division, dictate which toxin plasmid combinations can coexist within the same bacterial cell. We dissected the recognition steps between the DNA-binding ParMRC component, ParR, and the plasmid-derived centromere, parC. Our data suggested a mechanism by which plasmids encoding ParMRC systems from the same family are incompatible, whereas plasmids encoding ParMRC systems from distinct families are compatible. This work provides insight into how these cells can maintain multiple highly similar toxin plasmids, which is a critical first step in understanding how to limit the disease-causing potential of C. perfringens.

Keywords

plasmid partitioning, plasmid maintenance, plasmid incompatibility, Clostridium perfringens, ParR, parC, surface plasmon resonance, DNA binding, analytical ultracentrifugation
ParM, an actin-like ATPase that forms filaments in the presence of ATP or GTP; and ParR, a DNA-binding adaptor protein that binds to parC (2–6). ParMRC systems stabilize the inheritance of plasmids by positioning them on either side of the cell septum prior to cell division.

ParR proteins are typically ribbon-helix-helix proteins that bind to direct repeats within parC, either as a dimer or as a dimer of dimers (5, 7–10). The parC centromere usually consists of a series of direct repeats upstream of the parM gene; however, its precise genetic structure differs between plasmids. Binding of ParR acts to seed the formation of a higher-order solenoid-shaped structure, termed the segrosome, where the DNA wraps around ParR, leaving a core of ParM interaction sites (9, 10). Polymerizing ParM filaments then link the ParR-parC complexes of two sister plasmids and push them to either cell pole (2, 3, 11–13). The initial step, in which ParR recognizes and interacts with parC, is important in determining partition specificity between plasmids.

Plasmid incompatibility generally occurs when two coresident plasmids encode the same essential replication or partitioning machinery (14). Most studies to date have focused on the partition specificity and incompatibility mediated by type I (ParABS) partitioning systems (15–19); there is only limited evidence that partition-mediated incompatibility can also be facilitated by ParMRC-like partitioning systems (19, 20).

In this study, we focused on partition-mediated incompatibility in Clostridium perfringens, a Gram-positive pathogen. In humans and animals, C. perfringens produces an extensive range of toxins, which it uses to cause diseases that range from mild food poisoning to often fatal infections such as clostridial myonecrosis, enteritis, and enterotoxemia (21). Most C. perfringens toxins are encoded on large, low-copy-number, conjugative plasmids (22) that are similar to the tetracycline resistance plasmid pCW3 (22–29). These plasmids have approximately 35 kb of sequence similarity that includes the tcp conjugation locus and genes involved in replication, regulation, and stable plasmid maintenance (Fig. 1) (23, 24, 28, 30–33). Even though these plasmids have similar replication regions, including a highly conserved replication protein, C. perfringens strains frequently carry up to five discrete plasmids (24, 34). This phenomenon is typified by the avian necrotic enteritis isolate EHE-NE18, which stably maintains three large, closely related conjugative plasmids with Rep proteins that have 98% amino acid sequence identity (24, 34).

Bioinformatic analysis has revealed the presence of at least 10 families of ParMRC partitioning systems (ParMRCa to ParMRCj) in these pCW3-like plasmids. The ParM components have >90% amino acid sequence identity within a family and 15 to 54% amino acid sequence identity between families, and the ParR and parC components show a similar trend (34). A representative of the ParMRCa family was shown to be a true partitioning system, as addition of this partitioning system to an unstable minireplicon was sufficient to stabilize its inheritance in Escherichia coli (35). Strains of C. perfringens do not usually carry plasmids that encode the same ParMRC partitioning system (24, 28, 34), which suggests that these plasmids have evolved different partition specificities to ensure that they are stably maintained within a single C. perfringens cell.

We have shown that pCW3-like plasmids with identical partitioning systems cannot be maintained in a single cell without selection, whereas plasmids with ParMRC
systems from different families are stably maintained in *C. perfringens* cells (36). This finding suggested that differences in ParMRC plasmid partitioning systems were responsible for determining plasmid incompatibility between similar replicons and dictated which plasmid combinations could coexist in an isolate. In this study, we have utilized surface plasmon resonance (SPR) and analytical ultracentrifugation (AUC) to demonstrate that differences in the ParR and parC components of these partitioning families are reflected in their binding specificity, providing the essential biochemical evidence for the critical role of the ParMRC system in determining plasmid compatibility in *C. perfringens*.

**RESULTS**

Identification of the pCW3 ParRC binding site. The recognition steps between ParM, ParR, and parC components both within and between different families of parMRC systems are likely to be key drivers in determining the specificity of the partition reaction and therefore plasmid incompatibility in *C. perfringens*. The ParR-parC interaction is of particular interest because this is the first recognition step in the partitioning reaction (8, 10, 11) and is responsible for the incompatibility phenotype in some other plasmids (20).

SPR was employed to interrogate ParR-parC interactions. We first chose to examine the interaction between ParRC and parCC from pCW3, as pCW3 is the best-characterized conjugative antimicrobial resistance plasmid in *C. perfringens* (30). To perform SPR, a recombinant His6-tagged ParRC(pCW3) protein was expressed in *E. coli* and purified (see Fig. S1 in the supplemental material). A series of overlapping oligonucleotide fragments were designed (37) based on the 192-bp parCC region of pCW3 (Fig. 1). These oligonucleotides were annealed to produce a fragment array consisting of 18 double-stranded parCC fragments (designated C1 to C18) (Fig. 2A). The stability and specificity of the ParR-parC interaction were assessed by challenging each parCC fragment with ParRC(pCW3) (Fig. 2B and C). Strong interactions (a binding stability value of >100 response units [RU]) between ParRC(pCW3) and fragments C1 (256 RU), C5 (249 RU), C6 (282 RU), C11 (154 RU), C12 (348 RU), C15 (217 RU), and C16 (311 RU) were observed. Weak interactions (a stability value between baseline and 100 RU) were also noted for fragments C2 (54 RU), C7 (9 RU), C13 (48 RU), and C14 (42 RU).

The strong interactions that were observed between the parCC(pCW3) fragments and ParRC were mapped to the parCC(pCW3) nucleotide sequence, which showed that binding corresponded with the presence of four conserved 17-bp direct repeats (5′-AAACATCACAATTTTAC-3′).

Subsequently, a series of mutated parCC(pCW3) (C1) fragments were constructed to assess the importance of the 17-bp repeat to ParRC(pCW3) binding. Three altered fragments were constructed in which the cytosine and thymine bases in the 17-bp repeat were replaced with adenine (Fig. 3A), resulting in the generation of the fragments C1-5′', C1-3′', and C1-delta, which had four nucleotide changes, five nucleotide changes, and nine nucleotide changes in the 17-bp repeat, respectively. Analysis of the interaction between ParRC(pCW3) and these fragments revealed that any of these changes to the 17-bp repeat led to loss of ParR binding (Fig. 3A). The SPR results also indicated that a single fragment with the conserved parCC repeat was sufficient for ParRC binding.

AUC sedimentation velocity experiments were used to obtain insight into the multimeric state of ParRC in solution. The interaction between ParRC(pCW3) and the parCC(pCW3) fragment C5 was chosen for interrogation, as the C5 fragment had a centrally located direct repeat and showed strong binding to ParRC(pCW3) by SPR. The results showed that ParRC(pCW3) primarily sedimented as a single species, with a sedimentation coefficient ($s_{20,w}$) of 3.1 S (Fig. 2D), which corresponded to a molecular mass of 48 kDa (Fig. 2E). The molecular mass of His6-tagged ParRC(pCW3) as predicted from the amino acid sequence is 10.9 kDa, suggesting that ParRC(pCW3) exists as a tetramer in solution. The parCC(pCW3) C5 fragment sedimented as a single species, with a sedimentation coefficient of 2.7 S (Fig. S2A). When ParRC(pCW3) and parCC(pCW3) C5 were combined prior to centrifugation, a distinct shift in sedimentation coefficient to 4.2 S was observed (Fig. S2A), which was consistent with binding in a 1:1 ratio of
ParRC(pCW3) complex (four molecules) to each parCC(pCW3) binding site. Note that samples that are subjected to AUC are detected using UV light. However, ParRC does not contain any tryptophan residues and therefore fluoresces poorly when exposed to UV light. To compensate for this limitation, AUC was conducted using much higher concentrations of ParRC than were used in SPR. At these concentrations (>25 μM), ParRC also interacted with a nonspecific DNA control [parCC(pCW3) (C9)] (Fig. S2A). These results confirmed that ParRC(pCW3) and parCC(pCW3) (C5) could interact in solution, which was consistent with the results obtained via SPR but showed that ParRC(pCW3) can also interact nonspecifically at high concentrations. In addition, the stoichiometry of binding at high concentration may not reflect physiologically relevant complexes; we therefore drew upon our SPR binding data to determine the stoichiometry of binding between 0.1 μM ParRC and a parC fragment containing the predicted binding site (Table S6). SPR showed that the association between ParRC(pCW3) and bound parCC(pCW3) (C1, C5, C6, C11, C12, C15, and C16) was approximately 2:1 (ParR

**FIG 2** ParR.C(pCW3) binds to a cognate parC.C(pCW3) sequence. (A) Schematic of the parC.C(pCW3) fragment array, which consists of 30-bp fragments that overlap by 20 bp; direct repeats are indicated above the fragment array in red. (B) Representative ParR.C(pCW3) binding to the parC.C(pCW3) fragment array as determined by SPR. The first instance of C1 on the graph indicates a no-ParR control. Binding stability measurements were recorded 10 s after the end of sample injection. (C) Representative SPR binding curves for ParR.C(pCW3) and parC.C(pCW3) fragments. ParR.C(pCW3) plus the C3 binding curve is shown in blue, and ParR.C(pCW3) plus the C12 binding curve is shown in red. AUC sedimentation velocity experiments were also conducted on ParR.C(pCW3), parC.C(pCW3) fragment C5, and ParR.C(pCW3) and parC.C(pCW3) fragment C5 in combination. (D) Continuous sedimentation coefficient distribution [c(s)] as a function of normalized sedimentation coefficient [s_{20,W}] for ParR.C(pCW3). (E) Continuous mass distribution c(M) distribution as a function of molecular mass for ParR.C(pCW3).
to parC) except for the fragments C11 and C15, which do not have nucleotides immediately downstream of the direct repeat. This result suggests that interaction between a ParR dimer and its cognate binding site occurs when downstream context is provided.

To reconcile the differences in binding specificities observed between the SPR results and the AUC data, we performed electrophoretic mobility shift assays (EMSA) with ParRC(pCW3), parCC(pCW3) (C5), and parCC(pCW3) (C9). A specific shift was observed when ParRC was mixed with labeled C5 DNA at a ratio of 1 pmol to 1 pmol or 1 pmol to 4 pmol, compared to a no-protein control, which showed no shift (Fig. S2B). Similarly, when the unlabeled nonspecific inhibitor DNA parCC(pCW3) (C9) was included in the reaction in

FIG 3: ParRC(pCW3) and ParRB(pJIR4165) bind to direct repeats within cognate parC sites. (A) Items 1 to 4 show an alignment of the mutant parC fragments (C1-5', C1-3', and C1-delta) compared to parC (C1). SPR data of ParRC(pCW3) interaction with parC mutant fragments are shown in the graph in green (0.1 μM) and light green (0.05 μM). (B) Items 1 to 5 show an alignment of the mutant parC fragments (B17-5', B17-3', B17-delta, and B17-deltamiddle) compared to parC (B17). SPR data of ParRB(pJIR4165) interaction with parC mutant fragments are shown in the graph in blue (0.1 μM) and light blue (0.05 μM). All binding stability measurements were recorded 10 s after the end of sample injection.
excess (200×), a specific shift between ParR(pCW3) and the labeled parCC(pCW3) (C5) DNA was observed (Fig. S2B). In contrast, upon the addition of unlabeled specific competitor parCC(pCW3) (C5) DNA, the specific shift between ParR(pCW3) and labeled parCC(pCW3) (C5) was disrupted (Fig. S2B).

**ParR homologues cannot bind to noncognate parC centromeres from a different phylogenetic ParMRC family.** To determine if the interaction of ParR and parC components is ParMRC family specific, two more ParR and parC families were included in the SPR analysis. ParRb from pJIR4165 and ParRd from pJIR3118 have 11% and 26% amino acid sequence identity to ParRc(pCW3), respectively, and were expressed and purified (Fig. S1). In addition, parCB(pJIR4165) and parCD(pJIR3118) fragment arrays were synthesized to yield fragments B1 to B25 and D1 to D21 (Fig. 4A); these regions, respectively, have 45% and 47% nucleotide sequence identity to parCC(pCW3) (Table S1). ParRb(pJIR4165), ParRc(pCW3), and ParRd(pJIR3118) were tested against each parC fragment array [parC(pJIR4165), parC(pCW3), and parC(pJIR3118)] in separate SPR experiments (Fig. 4).

The results showed that each ParR homologue bound only to its cognate parC fragment array. ParRb(pJIR4165) bound to 12 parCB(pJIR4165) fragments, with the strongest binding (binding stability value of >300 RU) to fragments B2 (383 RU), B17 (368 RU), and
The parCC (pCW3) site had a clear correlation between binding and the direct-repeat structures, but the parCB (pJIR4165) region was more complex. The parCB (pJIR4165) site consists of several different direct repeats and two inverted-repeat structures, and many of these structures overlap. Therefore, mapping of ParRB (pJIR4165) binding to the parCB (pJIR4165) region did not indicate a clear ParRB (pJIR4165) binding site. The fragments that displayed the highest SPR response were aligned using Clustal Omega to identify conserved sequences that were required for ParRB (pJIR4165) binding (Fig. 5B). Two imperfect 8-bp direct repeats that were separated by 3 bp were identified in each fragment (Fig. 5B). Several mutated parC (pJIR4165) (B17) fragments then were constructed to assess the importance of these 8-bp repeats, and the spacing between them, to ParRB (pJIR4165) binding (Fig. 3B). Fragments were constructed that had the guanine, cytosine, and thymine bases in either the 5′ or the 3′ 8-bp repeat replaced with adenine, and in another fragment, the 3-bp spacing between the repeats was deleted (Fig. 3B). Analysis of ParRB (pJIR4165) binding to these fragments revealed that these changes to the B17-3′ 8-bp repeat (GATAAATC) resulted in a loss of binding. In contrast, replacement of the B17-5′ repeat with adenines resulted in a reduced level of ParRB binding. Finally, removal of the 3 bp between the two repeats showed a response comparable to that for the B17-3′ replacement fragment, i.e., loss of binding. This result demonstrated that this spacing region is important for the recognition and binding of ParRB to parCB (Fig. 3B).

ParRC (pCW3) was tested against the parCB (pJIR4165) and parCD (pJIR3118) fragment arrays and showed no interaction with these noncognate sequences (Fig. 4C). SPR analysis of the parC (pJIR3118) fragment array with its cognate ParRD (pJIR3118) protein showed strong binding stability values (>100 RU) with fragments D3 (225 RU), D4 (232 RU), and D5 (232 RU).

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**FIG 5** Sequence alignment of parC fragment that interact with ParR. The parC fragments that each ParR homologue interacted with were aligned using Clustal Omega to identify conserved binding sites; the predicted binding site is shown in bold, and identical residues are indicated by the asterisks. (A) parC (pCW3) fragment alignment; (B) parC (pJIR4165) fragment alignment; (C) parC (pJIR3118) fragment alignment.
RU), D9 (213 RU), D10 (270 RU), D11 (236 RU), D12 (250 RU), and D13 (187 RU) and weaker interactions (below 100 RU) with eight other oligonucleotide fragments (Fig. 4D). Inspection of the parCD (pJIR3118) region revealed several different AT-rich direct- and inverted-repeat structures.

In contrast to the other ParR proteins, ParRD proteins have a high pI value (~9 compared to ~4 to 5), and therefore, these proteins may bind more promiscuously to DNA than other ParR proteins. These nonspecific interactions were minimized by adding the blocking agent dextran to the SPR sample buffer; however, mapping of the ParRD(pJIR3118) interactions did not give a clear indication of the specific ParRD binding site. To further resolve the ParRD binding site, an SPR footprinting approach was used. A fragment that was composed of parCD fragments D3 and D4 and a series of sequential deletion derivatives based on this fragment with 2-bp deletions from either the 5’ or 3’ end were constructed (D0, DLHS1 to -12, and DRHS1 to -12) (Fig. 6). The ability of ParRD to bind to these fragments was tested, and the results showed that ParRD binding was greatly reduced.

**FIG 6** parCD(pJIR3118) SPR footprinting reveals ParRD(pJIR3118) binding site. Fragments with 2-bp deletions of the fragment D3 plus 4 from either right-hand side or 5’ (RHS) or left-hand side or 3’ (LHS) were constructed and tested for the ability to bind ParRD(pJIR3118). Oligonucleotide name and nucleotide sequence are indicated in the table. Relative binding response is indicated for each fragment by the values in column three and on the graph in orange. All binding stability measurements were recorded 10 s after the end of sample injection.

| Oligo | Sequence | Binding |
|-------|----------|---------|
| D34   | GAATAATTAAATAATCAGAATATATTTAATTCGTC | 570.8   |
| DLHS1 | AATAATTAAATAATCAGAATATATTTAATTCGTC | 557.1   |
| DLHS2 | AATAATTAAATAATCAGAATATATTTAATTCGTC | 503.3   |
| DLHS3 | TTAATTAAATAATCAGAATATATTTAATTCGTC | 528.7   |
| DLHS4 | TTAATTAAATAATCAGAATATATTTAATTCGTC | 466.2   |
| DLHS5 | AATATTAATCAGAATATATTTAATTCGTC | 465.6   |
| DLHS6 | AATATTAATCAGAATATATTTAATTCGTC | 373.8   |
| DLHS7 | AATATTAATCAGAATATATTTAATTCGTC | 136.1   |
| DLHS8 | TTAATTAAATAATCAGAATATATTTAATTCGTC | 221.5   |
| DLHS9 | TTAATTAAATAATCAGAATATATTTAATTCGTC | 91.4    |
| DLHS10| AATAATTAAATAATCAGAATATATTTAATTCGTC | 70.3    |
| DLHS11| AATAATTAAATAATCAGAATATATTTAATTCGTC | 27.3    |
| DLHS12| AATAATTAAATAATCAGAATATATTTAATTCGTC | 5.1     |
| DRHS1 | GAATAATTAAATAATCAGAATATATTTAATTCGTC | 572.5   |
| DRHS2 | GAATAATTAAATAATCAGAATATATTTAATTCGTC | 565.2   |
| DRHS3 | GAATAATTAAATAATCAGAATATATTTAATTCGTC | 560.6   |
| DRHS4 | GAATAATTAAATAATCAGAATATATTTAATTCGTC | 546.5   |
| DRHS5 | GAATAATTAAATAATCAGAATATATTTAATTCGTC | 459.2   |
| DRHS6 | GAATAATTAAATAATCAGAATATATTTAATTCGTC | 510.3   |
| DRHS7 | GAATAATTAAATAATCAGAATATATTTAATTCGTC | 443.1   |
| DRHS8 | GAATAATTAAATAATCAGAATATATTTAATTCGTC | 378.2   |
| DRHS9 | GAATAATTAAATAATCAGAATATATTTAATTCGTC | 352.3   |
| DRHS10| GAATAATTAAATAATCAGAATATATTTAATTCGTC | 84.6    |
| DRHS11| GAATAATTAAATAATCAGAATATATTTAATTCGTC | 98.3    |
| DRHS12| GAATAATTAAATAATCAGAATATATTTAATTCGTC | 34.7    |
when the AT-rich sequence 5'-ATAATATCAA was disrupted, indicating that this sequence is important for binding. Mapping this sequence to other strong binding fragments resulted in the identification of a partially conserved AT-rich ParR binding sequence (Fig. 5C).

ParR\textsubscript{p} (pJIR3118) did not interact with the par\textsubscript{C}\textsubscript{p} (pJIR4165) fragment array and showed only very weak interactions with most of the fragments from the par\textsubscript{C}\textsubscript{p} (pCW3) array (stability values between 5 and 15 RU above baseline). These interactions are likely to be nonspecific, as a low level of binding was observed for all fragments, including the reusable DNA capture technique (ReDCaT) control fragment. The non-specific interactions were minimized by the addition of dextran to the SPR sample buffer, which had no effect on binding to the par\textsubscript{C}\textsubscript{p} (pJIR3118) fragments. Overall, these results highlight the specificity of the ParR-par\textsubscript{C} interactions, where ParR homologues only bind to their cognate par\textsubscript{C} component and have either no interaction or very weak interfamily interactions.

ParR homologues recognize and bind noncognate par\textsubscript{C} fragment arrays from the same ParMRC family. Our earlier work suggested that ParMRC components from the same family would be able to interact with one another, thus leading to interference with the partition process and plasmid incompatibility (36). To provide biochemical evidence for this hypothesis, three different ParR homologues (ParR\textsubscript{p}, ParR\textsubscript{c}, and ParR\textsubscript{d}) from the \textit{C. perfringens} strain JGS1987 were expressed, purified (Fig. S1), and used to assess their capacity to facilitate intrafamily interactions. There is an unpublished whole-genome shotgun sequence available for strain JGS1987 (GenBank accession number: ABDW00000000), and it was chosen for analysis because an earlier bioinformatic survey revealed that this strain was particularly rich in parMRC genes (34). The JGS1987 sequence contains seven different par\textsubscript{M} alleles, which suggests that there may be seven potential plasmids present in this strain. Since these plasmid sequences had not been closed or given plasmid names, each putative plasmid was designated based on the strain of origin and the parMRC genes associated with that contig, yielding pJGS1987B, pJGS1987C, pJGS1987D, etc. The JGS1987 ParR\textsubscript{p}, ParR\textsubscript{c}, and ParR\textsubscript{d} homologues have 96%, 96%, and 95% amino acid identity to the equivalent ParR\textsubscript{p} (pJIR4165), ParR\textsubscript{c} (pCW3), and ParR\textsubscript{d} (pJIR3118) proteins (Table S1) (38). The corresponding JGS1987 par\textsubscript{C} regions also show high levels (82% to 91%) of nucleotide sequence identity to their equivalent homologues (Table S1 and Fig. S3). We postulated that the respective JGS1987-derived ParR proteins would cross-react with par\textsubscript{C} arrays from other members of the same ParMRC family. To examine this hypothesis, we tested the existing suite of par\textsubscript{C} fragment arrays with the purified ParR homologues from JGS1987.

The results showed that the JGS1987 ParR homologues interacted with noncognate par\textsubscript{C} fragment arrays from the same ParMRC family but not with noncognate par\textsubscript{C} fragments from different families (Fig. 7). ParR\textsubscript{p} (pJGS1987B) interacted with par\textsubscript{C}\textsubscript{p} (pJIR4165) with a binding pattern comparable to that of ParR\textsubscript{p} (pJIR4165) (Fig. 7A). Strong binding stability (>200 RU) scores were recorded for interactions between ParR\textsubscript{p} (pJGS1987B) and par\textsubscript{C}\textsubscript{p} (pJIR4165) fragments B1, B2, B3, B6, B8, B9, B10, B17, B18, B20, B21, B22, and B25. Weaker binding stability scores were seen for fragments B4, B7, B11, B16, and B23.

Similarly, ParR\textsubscript{c} (pJGS1987C) interacted only with par\textsubscript{C}\textsubscript{c} (pCW3), with the same binding pattern as observed for ParR\textsubscript{c} (pCW3) (Fig. 7B). High binding stability (>200 RU) scores were recorded for interactions between ParR\textsubscript{c} (pJGS1987C) and par\textsubscript{C}\textsubscript{c} (pCW3) fragments C1, C5, C6, C11, C12, and C15. Weaker binding stability scores were recorded for C2, C13, and C14.

ParR\textsubscript{d} (pJGS1987D) only interacted with its noncognate, but intrafamily, array from par\textsubscript{C}\textsubscript{d} (pJIR3118) (Fig. 7C). Strong binding stability scores were recorded for interactions between ParR\textsubscript{d} (pJGS1987D) and par\textsubscript{C}\textsubscript{d} (pJIR3118) fragments D3, D4, D9, D10, D11, D12, D13, and D19, whereas weaker binding stability scores were recorded for fragments D2, D5, D14, D16, D17, D18, and D20. Representative binding curves for each ParR-par\textsubscript{C} interaction pair are presented in Fig. S4. These data showed that ParR homologues interacted with noncognate par\textsubscript{C} fragments from the same phylogenetic ParMRC family, thus confirming a subset of the bioinformatically derived phylogenetic groups of these homologues.
DISCUSSION

In this study, we have demonstrated that ParR homologues from the pCW3 family of conjugative C. perfringens plasmids specifically recognize and bind to their cognate parC sites, providing biochemical evidence for the biological relevance of the phylogenetic ParMRC families that were previously identified (34). DNA binding studies showed that ParR proteins interacted with sequences within a centromeric parC site from the same ParMRC family but could not interact with a noncognate parC site from a different ParMRC family. We also demonstrated that ParR proteins can bind to noncognate parC sites from the same ParMRC family. These findings are consistent with our previous phenotypic analysis of ParMRC-encoding plasmids in C. perfringens, where plasmids from the same partitioning family were unable to be maintained in a single C. perfringens isolate in the absence of selection (36). These combined data provide clear experimental evidence that variation in the ParMRC partitioning systems represents a major molecular mechanism by which native C. perfringens isolates can maintain multiple closely related plasmids in the same cell.

All ParR proteins characterized to date bind to directly repeated sequences; however, the repeats they interact with vary between plasmid systems. For example, ParR from the E. coli plasmid R1 requires a minimum of two 11-bp repeats for binding (11), ParR from pB171 (E. coli) binds two 10-bp direct repeats upstream of parM (39), and ParR from the Staphylococcus aureus plasmid pSK41 binds to 20-bp repeats (10).

The direct repeats in the C. perfringens parC sites differ substantially between families with respect to both their nucleotide sequence and their spacing within the centromere. ParR binding correlated with four 17-bp direct repeats within the parC region. These repeat structures are conserved between parC regions of different plasmids, supporting the assertion that ParR is able to recognize and bind to these sites. In contrast, the ParR and ParRb binding sites were more difficult to delineate because there were multiple direct and inverted repeat structures within the parC and parC regions.

Our findings support the hypothesis that the inability of ParR proteins to discriminate between closely related parC sites is responsible for previously observed ParMRC-mediated plasmid incompatibility (36). The consequence would be the incorrect linkage of two heterologous plasmids, eventually leaving distinct populations of daughter cells each containing only one of these plasmids (14, 17, 18, 40). Although the heterologous pairing model is not favored for type I partitioning-mediated incompatibility (16, 18), there is evidence that suggests that this model could explain ParMRC-based plasmid incompatibility. For example, ParR from R1 is capable of...
linking replicons before partitioning and promiscuous binding of ParR from pB171 is responsible for plasmid incompatibility (8, 20).

In this study, we have demonstrated that the interaction between ParR and parC is important for plasmid incompatibility; however, there is a second key recognition step in the partition reaction between the filament-forming protein ParM and the ParR-parC complex. ParM falls into the same phylogenetic groups as ParR and parC; therefore, it is likely that ParM has a specificity profile similar to that of ParR and parC. We postulate that ParM will interact with ParR-parC complexes from the same family but will not recognize noncognate ParR-parC complexes from other families. Further studies will aim to characterize the interaction between ParM and ParR in *C. perfringens* and determine whether this recognition step follows a pattern similar to that of the ParR-parC interactions outlined in this study.

For technical reasons already outlined, the AUC experiments were conducted using high concentrations (25 μM) of ParR<sub>pCW3</sub>. Analysis of our sedimentation velocity data showed that ParR<sub>pCW3</sub> formed a tetrameric complex in solution; however, the concentration of ParR<sub>pCW3</sub> used (25 μM) is unlikely to reflect a physiologically relevant level of ParR protein within the cell. Dissection of the SPR binding data between ParR<sub>pCW3</sub> and parC<sub>pCW3</sub> fragments that contain a predicted binding site suggested a 2:1 association of ParR to parC. Further experiments are required to confirm the oligomeric state of ParR<sub>pCW3</sub>. Furthermore, upon the addition of parC<sub>pCW3</sub> fragments, a higher sedimentation coefficient was observed. At this concentration, ParR<sub>pCW3</sub> interacted with the parC<sub>pCW3</sub> C9 fragment, despite the absence of the 17-bp direct repeat, suggesting that at high concentrations, ParR<sub>pCW3</sub> is capable of binding DNA nonspecifically. EMSA confirmed that ParR<sub>pCW3</sub> binding to parC<sub>pCW3</sub> was nonspecific and that ParR<sub>pCW3</sub> binding to parC<sub>pCW3</sub> (C5) was a specific interaction at lower concentrations (1 μM).

These data are consistent with previous structural studies of ParR proteins from pSK41 and pB171 (9, 10), which form tight dimers in solution and bind cooperatively to the DNA major groove within the parC centromere (5, 8–11). Once bound to parC, ParR forms a segrosome, where contacts between each ParR dimer are made, ultimately resulting in the formation of a dimer of dimers.

Replicon coevolution appears to be widespread in *C. perfringens*: different isolates often carry closely related plasmids with different ParMRC partitioning systems (22, 24, 28). For example, the avian necrotic enteritis strain EHE-NE18 has three plasmids that have similar replication proteins but different ParMRC system families (ParMRC<sub>A</sub>, ParMRC<sub>B</sub>, and ParMRC<sub>C</sub>) (24). Based on the ParR<sub>A</sub>, ParR<sub>B</sub>, and ParR<sub>C</sub> binding data reported here, and the previous genetic studies (36), it is concluded that to ensure that each plasmid is segregated independently, these ParMRC systems have coevolved to carry different partition specificities.

The evolution of multiple ParMRC partition specificities in *C. perfringens* cells is reminiscent of the evolution of independent ParABS systems in *Burkholderia cenocepacia*. The pathogenic *B. cenocepacia* strain J2315 maintains three chromosomes and a large, low-copy-number plasmid (41). The type I (ParABS) partitioning systems of these replicons have coevolved to become distinct so that each replicon is partitioned independently (41–44). Likewise, *Rhizobium leguminosarum* bv. trifolii RepB (ParB homologue) proteins discriminate between similar parS centromeres to independently segregate and maintain a chromosome in addition to four plasmids (45). Unlike *B. cenocepacia* and *R. leguminosarum*, where the selection pressure to maintain multiple chromosomes and plasmids seems to have driven the coevolution of separate partition specificities, the selective pressure that has resulted in the generation of so many parMRC alleles in these conjugative *C. perfringens* plasmids remains unclear. One explanation may be that the ParMRC systems act as a means of competitive exclusion. It can be envisioned that upon entry into a new cell via conjugation, pCW3-like plasmids could displace resident plasmids that encode similar partitioning systems, thereby generating two distinct bacterial subpopulations, each carrying a single plasmid. In addition, the plasmid-borne toxin and antibiotic resistance genes
TABLE 1  Bacterial strains and plasmids used in this study

| Strain or plasmid | Description* | Reference or source |
|-------------------|--------------|---------------------|
| **C. perfringens** |  | |
| CN1020            | Type D isolate carrying the etx gene on pJIR3118 | 53 |
| JGS1987           | Type E isolate carrying iap/ibp, cpe, lam, and cpb2 toxin genes | GenBank accession no. ABDW00000000 |
| JIR4195           | JIR325(pCW3) Tc’ | 53 |
| CN4003            | Type D isolate carrying etx, cpe, cpb2, and lam toxin genes | 54 |
| **E. coli**       |  | |
| BL21(DE3)         | fhuA2 [lom] ompT gal (λ DE3) [dcm] ΔhsdSΔλ DE3 = λ sBamH1O  
|                   | ΔEcoR-B int[: tacl:PlacUV5::T7 gene1] i21 Δnin5 | New England Biolabs |
| C41(DE3)          | BL21(DE3) derivative | 55 |
| C43(DE3)          | BL21(DE3) derivative | 55 |
| **Plasmid**       |  | |
| pCW3              | Isolated from CW92; 47 kb; Tc’; parMRCC(pCW3) | 56 |
| pET22b (+)        | T7 promoter expression vector; IPTG inducible; adds C-terminal His6 tag; Amp’ | Novagen |
| pJGS1987B         | Plasmid from JGS1987; carries parMRCC(pJGS1987B) | GenBank accession no. ABDW01000017 |
| pJGS1987C         | Plasmid from JGS1987; carries parMRCC(pJGS1987C) | GenBank accession no. ABDW01000012 |
| pJIR3118          | 48-kb etx-bearing plasmid from CN1020; carries parMRCC(pJIR3118) | 53 |
| pJIR4165          | CPE-encoding plasmid isolated from CN4003 (100 kb); carries parMRCC(pJIR4165) | V. Adams, D. Lyras and J. I. Rood, unpublished data |
| pJIR4519          | pET22b (+)ΔNdeI/Xhol parR from pCW3 | This study |
| pJIR4767          | pET22b (+)ΔNdeI/Xhol parR from pJGS1987B | This study |
| pJIR4768          | pET22b (+)ΔNdeI/Xhol parR from pJGS1987C | This study |
| pJIR4769          | pET22b (+)ΔNdeI/Xhol parR from pJGS1987D | This study |
| pJIR4773          | pET22b (+)ΔNdeI/Xhol parR from pJIR4165 | This study |
| pJIR4820          | pET22b (+)ΔNdeI/Xhol parR from pJIR3118 | This study |

*pC’, tetracycline resistant; Amp’, ampicillin resistant. C. perfringens enterotoxin; etx, epsilon-toxin gene; lam, lambda toxin gene; iap and ibp, iota toxin genes; cpb2, beta2 toxin gene.

may result in the positive selection of these plasmids in certain environmental niches, providing a selective advantage for the host cell if it can maintain these closely related plasmids. There is most certainly more complexity involved in the incompatibility phenotype in C. perfringens, since other factors, such as the timing of plasmid replication, the plasmid copy number, and plasmid replication initiation and regulatory proteins, may play at least some role in determining whether two replicons are incompatible or are maintained in the same cell, as in other bacteria (14, 15, 18, 46).

In conclusion, we have shown that interaction between the ParMRC partitioning components ParR and parC occurs only between members of the same phylogenetic family. These results provide biochemical insight into the basis of C. perfringens plasmid incompatibility and explain how multiple plasmids with similar replicons can be maintained within a single C. perfringens isolate.

MATERIALS AND METHODS

Plasmids, bacterial strains, and culture conditions. All C. perfringens strains, Escherichia coli strains, and plasmids used in this study are listed in Table 1. All E. coli strains were grown on 2x yeast-tryptone (2YT) agar supplemented with 100 μg/mL of ampicillin and incubated at 37°C overnight. E. coli expression strains were grown in either 2YT broth or autoinduction medium (AIM) (47, 48).

Construction of ParR expression vectors. The parR gene from pCW3 was codon optimized for expression in E. coli, synthesized by GenScript, and cloned into the EcoRV site of pUC57-Kan. Codon-optimized parR, then was subcloned into the NdeI/Xhol sites of pET22b (+); parR(pJIR3118) was PCR amplified from CN1020 genomic DNA (gDNA) isolated as described previously (49) and cloned into the NdeI/ Xhol site of pET22b (+) for expression. parR(pJIR4165), parR(pJGS1987B), parR(pJGS1987C), and parR(pJGS1987D) were codon optimized and synthesized before being cloned into pET22b (+) NdeI/ Xhol sites by GenScript.

ParR expression and purification. ParR proteins with C-terminal His6 tags were expressed using E. coli strain C43(DE3), C41(DE3), or BL21(DE3). The cells either were grown at 28°C in autoinduction medium for 24 h before the temperature was lowered to 22°C for 6 h or were grown in 2YT broth at 37°C to an optical density at 600 nm (OD600) of 0.6 and induced with the addition of 0.1 mM IPTG (isopropyl-β-D-12
β-thiolacopyranoside for 3 h (Table S2). Cells were lysed using a cell disruptor (Avestin) (lysis buffer: 20 mM Tris (pH 7.9), 300 mM NaCl, 10% glycerol, 1 mg/mL of DNase I and Complete protease inhibitors (Roche)), and proteins were purified (Fig. S1) using TALON resin (Clontech) and eluted with the addition of increasing concentrations of imidazole (5 mM to 200 mM) in purification buffer (20 mM Tris (pH 7.9), 300 mM NaCl, 10% glycerol) and confirmed by Western blotting. All ParR proteins were buffer exchanged into buffer A (10 mM HEPES (pH 7.4), 300 mM NaCl, 3 mM EDTA, 0.05% Tween 20, 0.02% NaN₃) using a 3-kDa centrifugal filter (Amicon) before dilution to 0.1 μM. Independent preparations of each purified ParR protein were used as biological repeats for SPR.

Fragment array preparation for SPR experiments. parC fragment arrays were constructed as previously described (37) using the reusable DNA capture technique (ReDCaT). Briefly, the parC regions of pCW3 (192 bp), pJR3118 (230 bp), and pJR4165 (262 bp) were used as templates for the Perl overlapping oligonucleotide program (POOP). POOP produced a series of overlapping forward and reverse 30-bp oligonucleotides (20-bp overlaps). Reverse-strand oligonucleotides had a 20-bp 3′ sequence (5′-CCTACCTCCTAGCTCCTTGCT-3′) that was complementary to the ReDCaT sequence. The parC C1 and parC B17 mutant fragments and the parC D3-D4 footprinting oligonucleotides were constructed as described above (the ligands used in SPR experiments are listed in Table S3). Oligonucleotides were synthesized (Integrated DNA Technologies) at a concentration of 100 μM in IDE buffer (10 mM Tris, 0.1 mM EDTA (pH 8.0)). To construct fragments for SPR analysis, complementary oligonucleotides were mixed in a ratio of 1:2:1 (forward to reverse), annealed at 98°C for 10 min, and cooled for 30 min at room temperature. Fragments were then diluted to 0.5 mM in buffer A.

Surface plasmon resonance. SPR experiments were based upon the ReDCaT method as previously described (37) and conducted using the Biacore T200 system (GE Healthcare Life Sciences). All experiments were carried out on an S series Biacore sensor chip (GE Healthcare Life Sciences) with streptavidin preimmobilized to a carboxymethylated dextran matrix for capture of biotinylated interaction partners.

Prior to SPR, all four flow cells of the SA chip were washed three times with buffer containing 1 M NaCl and 50 mM NaOH. After washing and priming with buffer A, biotinylated ReDCaT linker (100 nM (5′-biotin-GCAGGGAGCTAGGTAGG-3′)) was immobilized to all four flow cells at 5 μL/min to a capture level of ~500 response units (RU). Subsequently, the chip was primed with buffer A, the ReDCaT complementary oligonucleotide (500 nM) was captured on flow cell 1, and parC ligands diluted in buffer A to a concentration of 500 nM were captured to flow cells 2 to 4 (parCf, parCco, and parCf fragments on flow cells 2, 3, and 4, respectively) to a density of approximately 200 RU under flow conditions (10 μL/min for 30 s). DNA capture levels are listed in Table S4. The first flow cell was used as a reference cell for subtraction of flow cell 2, 3, and 4. Each ParR protein (ParRb,pJR4165, ParRb,pJS1987B, ParRb,pCW3), ParRb (pJS1987B), and ParRb (pCW3) was diluted to a concentration of 0.1 μM in buffer A, and ParRb (pJR3118) was diluted in buffer A with 1 mg/mL of dextran to reduce nonspecific binding. Proteins were flowed through all four flow cells at 30 μL/min with 60 s of association and 60 s of dissociation. Binding stability measurements were recorded 10 s after the end of sample injection. All four flow cells of the chip were regenerated after each cycle using regeneration buffer (1 M NaCl and 50 mM NaOH) to leave only the biotinylated ReDCaT oligonucleotide. All experiments were conducted at 20°C. All SPR methods were programmed using Biacore T200 control software, and data were analyzed using Biacore evaluation version software version 2.0. The ParRb(pCW3)-parC, ParRb(pCW3) binding stoichiometry was calculated by dividing the background subtracted RU recorded for ParRb by the RU of immobilized DNA deposited on the sensor chip.

Analytical ultracentrifugation. Sedimentation velocity experiments were performed in an Optima analytical ultracentrifuge (Beckman Coulter) equipped with UV-visible (UV-Vis) scanning optics. ParRb(pCW3) was prepared at a concentration of 0.5 mg/mL with and without 0.1 mg/mL of parCf DNA (fragment C5). Reference (400 μL of buffer A without Tween 20) and sample (370 μL) solutions were loaded into double-sector cells with quartz windows. These cells were mounted in an An-50 Ti 8-hole rotor. Proteins and DNA were centrifuged at 40,000 rpm at 20°C, and radial absorbance data were collected at appropriate wavelengths (~280 nm) in continuous mode every 20 s. The partial specific volume (~H) of ParRb (0.7372), buffer density (1.0119 g/mL), and buffer viscosity (0.0104 poise [P]) were determined using the program SEDNTERP (50). The partial specific volume (~H) of parCf DNA (0.5500) was determined using UltraScan III (51). Data were fitted to continuous size distribution [c(s)] and continuous mass distribution [c(M)] models using the program SEDFIT (52). All sedimentation coefficient data were normalized to standard conditions at 20°C in water (s20,w) relevant hydrodynamic properties are listed in Table S5.

Electrophoretic mobility shift assay. Target 30-bp DNA fragments were generated by annealing forward and reverse oligonucleotides. All gel shift DNA was labeled with digoxigenin-11-ddUTP (DIG) at their 3′ termini with the DIG gel shift kit (Roche) as per the manufacturer’s instructions. Gel mobility shift assays were carried out using the DIG gel shift kit, 2nd generation (Roche). Reactions testing ParRb(pCW3) binding included 4 μL of binding buffer [100 mM HEPES (pH 7.6), 5 mM EDTA, 50 mM (NH₄)₂SO₄, 5 mM diethiothreitol (DTT), 1% (wt/vol) Tween 20, 150 mM (KCl) (Roche), 1 μg of poly(dI-dC)], 0.1 μg of poly(I-Lys), 1 pmol of DIG-labeled target DNA, 1 pmol (1:1) or 4 pmol (1:4) of His6-ParRb(pCW3), and sterile deionized water in a total volume of 20 μL. For reactions that tested ParRb(pCW3) binding specificity, 150 to 200 pmol of unlabeled parCf (pCW3) (C9) or unlabeled parCf (pCW3) (C5) DNA was added to reaction mixtures containing 1 pmol of DIG-labeled parCf (pCW3) (C5) DNA and 1 pmol of His6-ParRb. Reaction mixtures were incubated for 15 min at room temperature before the addition of gel loading buffer without bromophenol blue. Reaction mixtures were loaded immediately onto 10% (wt/vol) native 1 × TBE (22.3 mM Tris, 22.3 mM boric acid, 0.5 mM EDTA (pH 8.0)) polyacrylamide gels with a control lane containing gel loading buffer with bromophenol blue. Samples
were separated at 173 V for 40 min and then transferred onto Nylon+ membranes (Amersham Life Science, UK) by electroblotting with 1 x TBE (pH 8.0) at 100 V for 1 h. Following transfer, the membrane was UV cross-linked and chemiluminescent detection of DIG epitope was carried out as per the manufacturer’s instructions (Roche). Chemiluminescence was recorded using Bio-Rad Chemidoc imaging systems (Bio-Rad).

**SUPPLEMENTAL MATERIAL**

Supplemental material is available online only.

**FIG S1**, DOCX file, 0.6 MB.

**FIG S2**, DOCX file, 0.6 MB.

**FIG S3**, DOCX file, 0.1 MB.

**FIG S4**, DOCX file, 0.6 MB.

**TABLE S1**, DOCX file, 0.01 MB.

**TABLE S2**, DOCX file, 0.01 MB.

**TABLE S3**, DOCX file, 0.02 MB.

**TABLE S4**, DOCX file, 0.03 MB.

**TABLE S5**, DOCX file, 0.01 MB.

**TABLE S6**, DOCX file, 0.01 MB.

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