JOURNAL OF BACTERIOLOGY, July 2006, p. 4942–4951 Vol. 188, No. 13
0021-9193/06/$08.00+0 doi:10.1128/JB.00298-06

Functional Identification of Conjugation and Replication Regions of the Tetracycline Resistance Plasmid pCW3 from *Clostridium perfringens*

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Received 28 February 2006/Accepted 16 April 2006

Clostridium perfringens **causes fatal human infections, such as gas gangrene, as well as gastrointestinal diseases in both humans and animals. Detailed molecular analysis of the tetracycline resistance plasmid pCW3 from *C. perfringens* has shown that it represents the prototype of a unique family of conjugative antibiotic resistance and virulence plasmids. We have identified the pCW3 replication region by deletion and transposon mutagenesis and showed that the essential *rep* gene encoded a basic protein with no similarity to any known plasmid replication proteins. An 11-gene conjugation locus containing 5 genes that encoded putative proteins with similarity to proteins from the conjugative transposon Tn*916* was identified, although the genes' genetic arrangements were different. Functional genetic studies demonstrated that two of the genes in this transfer clostridial plasmid (*tcp*) locus, *tcpF* and *tcpH*, were essential for the conjugative transfer of pCW3, and comparative analysis confirmed that the *tcp* locus was not confined to pCW3. The conjugation region was present on all known conjugative plasmids from *C. perfringens*, including an enterotoxin plasmid and other toxin plasmids. These results have significant implications for plasmid evolution, as they provide evidence that a nonreplicating Tn*916*-like element can evolve to become the conjugation locus of replicating plasmids that carry major virulence genes or antibiotic resistance determinants.**

Conjugative plasmids are self-replicating molecules that encode their own transfer to recipient strains, usually by a type IV secretion apparatus. Conjugative plasmids from gram-positive bacteria, such as *Enterococcus*, *Staphylococcus*, and *Bacillus* spp., employ similar mechanisms (23); however, the absence of an outer membrane and the presence of a much thicker peptidoglycan layer necessitates a requirement for different conjugation complexes. Integrative conjugative elements (ICEs), or conjugative transposons, are also capable of encoding their own conjugative transfer, but these elements are not self replicating and require genomic integration for their stable maintenance. The best known ICEs are Tn*916* from *Enterococcus faecalis* and Tn*1545* from *Streptococcus pneumoniae*, but little is known about their mechanisms of conjugation (45). ICEs have been identified from many gram-positive bacteria, including the pathogenic clostridia. These elements include the Tn*916*-like conjugative element Tn*5397* from *Clostridium difficile* and the defective element CW459*tet*(M) from *Clostridium perfringens* (46).

The 47-kb tetracycline resistance plasmid pCW3 (49) carries a novel tetracycline resistance operon (56) and is the paradigm conjugative plasmid from *C. perfringens*. It is closely related to pIP401, which carries the same tetracycline resistance genes, and also carries Tn*4451*, an integrative mobilizable element that confers chloramphenicol resistance (5). Deletion of Tn*4451* from pIP401 (3) results in a plasmid that has a restriction profile identical to that of pCW3. Comparative restriction and hybridization analysis has shown that all conjugative tetracycline resistance plasmids from *C. perfringens* are either indistinguishable from pCW3 or have a large common region (3). In addition, there are large regions of similarity between pCW3 and the plasmids that carry the enterotoxin gene, *cpe* (12, 36).

The objective of this study was to determine if the region that was common between these plasmids was involved in conjugative transfer. We have identified the replication and conjugation regions of pCW3 and carried out comparative analyses. In this study, we report that pCW3 carries a unique conjugation region that is common to all conjugative *C. perfringens* plasmids and that several of the conjugation genes have putative products with similarity to conjugation proteins from Tn*916*.

MATERIALS AND METHODS

Bacterial strains and plasmids. The *C. perfringens* strains used in this study were wild-type tetracycline-resistant isolates from diverse sources. The recipients in conjugation experiments were either JIR325, a rifampin- and nalidixic acid-resistant derivative of strain 13 (33), or JIR4394, a streptomycin- and chlorate-resistant derivative of strain 13. *C. perfringens* strains were cultured at 37°C in TPG broth (48), brain heart infusion broth (Oxoid), FTG medium (Difco), or nutrient agar (47) supplemented with tetracycline (10 μg/ml), rifampin (20 μg/ml), nalidixic acid (20 μg/ml), chloramphenicol (10 μg/ml), thiamphenicol (10 μg/ml), or streptomycin (1 mg/ml). When required, 1% (vol/vol) saturated potassium chlorate was included. *C. perfringens* agar cultures were incubated in an atmosphere of 10% H_2–10% CO_2–80% N_2. The *Escherichia coli* host strain used was DH5α (Life Technologies) or EC300 (Epicenter), which was grown at 37°C in 2× YT medium (50) supplemented with ampicillin (100 μg/ml), erythromycin (150 μg/ml), or kanamycin (50 μg/ml). Plasmids are listed in Table 1.

Molecular techniques. *E. coli* plasmid DNA was isolated using alkaline lysis (QIAGEN). Crude *C. perfringens* DNA was extracted by resuspending cells from an agar culture in 100 μl of lysis buffer, boiling for 10 min, and centrifuging for 10 min at 11,000 × *g* at room temperature. The DNA in the supernatant was phenol:chloroform and chloroform extracted, isopropanol precipitated, washed with ethanol, and resuspended in 10 μl of H_2O. Purified *C. perfringens* DNA was obtained as described previously (48). PCR amplification used *Taq* DNA poly-

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TABLE 1. Origin and source of plasmids

Plasmid	Description	Source or reference
pCW3	Confers conjugative tetracycline resistance	49
pJIR15	pBR322 ΩpCW3(ClaI 9.8 kb)	1
pJIR16	pBR322 ΩpCW3(ClaI 12.2 kb)	1
pJIR17	pBR322 ΩpCW3(ClaI 10.4 kb)	1
pJIR18	pBR322 ΩpCW3(ClaI 9.9 kb)	1
pJIR26	pJIR27 ΔTn*4452*	3
pJIR32	pBR322 ΩpCW3(ClaI 5.0 kb)	1
pJIR936	pBR322 ΩpCW3(ClaI 9,744 bp) Ω1.2 kb BamHI, *erm*(B)	K. Koutsis and J. I. Rood, unpublished
pJIR1909	pCW3 10,358-bp ClaI (bp 41533–4628) + pCW3 9,744-bp ClaI (bp 4628–14372)	P. Johanesen and J. I. Rood, unpublished
pJIR2765	pJIR936 Δ5.2 kb HpaI	This study
pJIR2766	pJIR936 Δ8.8 kb EcoRV	This study
pJIR2767	pJIR936 Δ3.1 kb XbaI	This study
pJIR2768	pJIR2675 Δ1.2 kb NheI/SpeI	This study
pJIR2715	Base *C. perfringens* suicide vector, *erm*(Q)$^+$ *catP*$^+$ (inactivating Kn^r) *oriT*$^+$	C. Hennequin and J. I. Rood, unpublished
pJIR2898	pJIR2715 (Asp718/BamHI) ΩJRP1997/JRP1998 PCR product (Asp718/BamHI; 1,900 bp) (5′ *tcpH* fragment)	This study
pJIR2899	pJIR2898 (XhoI/SacI) ΩJRP1999/JRP2000 PCR product (XhoI/SacI; 1,996 bp) (*tcpH* suicide vector)	This study
pJIR2901	pJIR750 (Asp718/BamHI) ΩJRP2119/JRP2120 PCR product (Asp718/BamHI; 3,030 bp) (p*tcpH*$^+$ complementation vector)	This study
pJIR3023	pJIR2715 (XhoI/SacI) ΩJRP1995/JRP1996 PCR product (XhoI/SacI; 2,070 bp) (3′ *tcpF* fragment)	This study
pJIR3024	pJIR3023 (Asp718/BamHI) ΩJRP1993/JRP1994 PCR product (Asp718/BamHI; 1,981 bp) (*tcpF* suicide vector)	This study
pJIR3025	pJIR750 (Asp718/BamHI) ΩJRP2347/JRP2348 PCR product (Asp718/BamHI; 2,869 bp) (p*tcpF*$^+$ complementation vector)	This study
pMRS4969	pCPF4969 *cpe* Ω*catP*	12

merase (Roche) and a 0.5 μM concentration of each primer. Denaturation (94°C for 1 min), annealing (50°C for 2 min), and extension (72°C for 3 to 5 min) steps were carried out for 30 cycles. PCR products were purified using the QIAquick PCR purification kit before sequencing on an Applied Biosystems 3730S capillary sequencer.

The complete pCW3 sequence was determined on both strands, with the exception of the previously sequenced tetracycline resistance determinant (56). Templates for plasmid sequencing were existing (1) and newly constructed subclones of pCW3. Sequencing of PCR products was used to cross all restriction sites used in cloning. Sequence data were analyzed using Sequencher version 3.0 (Gene Codes Corporation), and potential genes were identified using GeneMarkS (11), in conjunction with the Sanger Institute freeware Artemis, release 6. Putative gene products were analyzed using PSI-BLAST (6, 7), TopPred (14), and PSORT Prediction (38). Sequences were aligned using ClustalW (28).

Construction of *C. perfringens* mutants by allelic exchange. To increase the frequency of double crossovers, the suicide plasmids contained ca. 2 kb of sequence upstream and downstream of the gene to be mutated. These regions were generated by PCR and then cloned sequentially into the *E. coli* vector pJIR2715, which contains genes encoding thiamphenicol and erythromycin resistance (C. Hennequin and J. Rood, unpublished data). The base pair (bp) 28,500 to 30,481 pCW3 region was cloned upstream of *erm*(Q), and the bp 32,648 to 34,718 region was cloned downstream of *erm*(Q) to generate the *tcpF* suicide vector pJIR3024. Similarly for *tcpH*, the regions bp 32,084 to 33,984 and bp 36,275 to 38,271 of pCW3 were cloned upstream and downstream, respectively, of *erm*(Q) to form pJIR2899.

The suicide vectors were independently introduced into JIR325(pCW3) by electroporation (52). DNA preparations of potential erythromycin-resistant/thiamphenicol-sensitive recombinants were tested by PCR and sequence analysis to confirm the replacement of the target gene with the *erm*(Q) cassette and loss of the suicide plasmid. For complementation studies, PCR products carrying the wild-type *tcpF* and *tcpH* genes were generated and cloned into the *C. perfringens-E. coli* shuttle vector pJIR750 to generate pJIR3025 and pJIR2901, respectively.

Conjugation. Matings on solid media were carried out as described previously (47, 48). Nutrient agar supplemented with tetracycline, streptomycin, and potassium chlorate was used to select for transconjugants when *C. perfringens* strain JIR4394 was used as the recipient. The efficiency of conjugative transfer is reported as the number of transconjugants/donor cell.

Transposon mutagenesis. Transposon mutagenesis with the EZ::TN In-Frame Linker Insertion kit (EPICENTRE), performed per the manufacturer's instructions, was used to analyze the target plasmid, pJIR2768. The in vitro reaction mixture was introduced into EC300 cells by electroporation, and kanamycin-resistant cells were selected. EZ::TN insertions were mapped and sequenced.

Nucleotide sequence accession numbers. GenBank accession numbers for pCW3 and the *tcp* regions of pJIR26 and pMRS4969 are DQ366035, DQ338471, and DQ338472, respectively.

RESULTS

Sequence analysis of pCW3. The complete nucleotide sequence of pCW3 was determined by a combination of sequence analysis of subclones and PCR fragments as well as primer walking. Where PCR fragments were used, the sequence was confirmed by sequencing at least two separate PCR products on both strands. pCW3 was shown to comprise 47,263 bp, with a G+C content of 27.6%, which is similar to that of the *C. perfringens* chromosome. Our analysis identified 51 open reading frames (ORFs) (Fig. 1 and Table 2).

Comparative analysis showed that the bp 16332 to 22432 region of pCW3 had 70% nucleotide sequence identity to a similar-size region (bp 51011 to 44615) of the 53-kb plasmid pCP13 from *C. perfringens* strain 13 (55). pCP13 carries a defective β2 toxin gene, *cbp2*, that is not present on pCW3. This region of pCW3 carried nine putative genes, with the seven largest genes having distinct homologues within pCP13 (Fig. 2). The region included the *cna* gene, whose product had similarity to a collagen adhesin from *Staphylococcus aureus* (39,

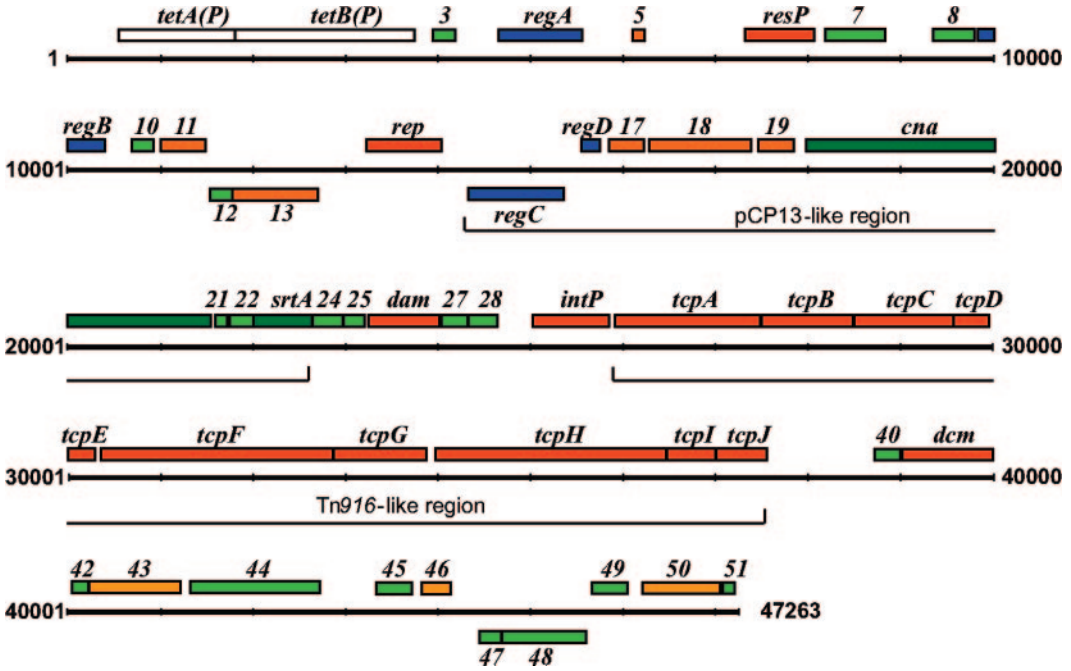

FIG. 1. Genetic organization of pCW3. The first T of the −35 box of the promoter for the *tetAB*(P) operon (24), which encodes tetracycline resistance, was designated nucleotide number 1. ORFs on the sense and complementary strands are indicated above and below the plasmid line, respectively. Genes with unknown functions are indicated by their designated pCW3 gene number, putative conjugation genes as *tcp* genes, and putative regulatory genes as *reg* genes. The labeled bars indicate regions with either similarity to a locus present on pCP13 or genes whose products have low-level similarity to Tn*916* conjugation proteins. Colors indicate the gene product's function: red, DNA metabolism (replication, recombination, DNA transfer, and modification); dark green, membrane and surface associated; yellow, miscellaneous metabolism; orange, conserved hypothetical; light green, unknown; white, antibiotic resistance; and blue, regulation.

40, 44, 57) (Fig. 1). Two potential methyltransferase genes (*dam* and *dcm*) were identified on pCW3, both of which have been identified on the conjugative *cpe* plasmid pCPF4969 and on another *cpe* plasmid, pCPF5603 (35, 36). The *dcm* gene is located in close proximity to the *cpe* gene on these plasmids and is present in other *cpe*$^+$ and non-*cpe*-carrying *C. perfringens* strains (35, 36).

Tn*4451* is inserted into the *cna* gene of pIP401. The well-studied element Tn*4451* (4, 9, 29, 30, 32) was originally identified on the pCW3-like plasmid pIP401 (34). It was postulated based on comparative restriction endonuclease analysis that pIP401 consisted of a pCW3 plasmid into which Tn*4451* had inserted (2). By comparing the nucleotide sequence of the left and right ends of the insertion site of Tn*4451* to the sequence of pCW3, the corresponding site within pCW3 to the insertion site of the element on pIP401 has now been identified at bp 21,329 in pCW3. This site is located within the *cna* gene, 67 codons from the stop codon. As pIP401 is still able to encode its own conjugative transfer, this result indicates that the terminal 67 amino acids of the putative Cna protein are not required for conjugation.

Replication of pCW3 involves a unique Rep protein. Annotation of the pCW3 sequence did not reveal any proteins with similarity to plasmid replication proteins. A functional genetic approach, therefore, was used to identify the replication region. It was initially shown that pJIR1909, a deletion derivative of pCW3 that encompassed a 20.1-kb (bp 41533 to 14372) region, could support its own independent replication in *C. perfringens* (P. Johanesen, D. Lyras, and J. Rood, unpublished data). To further delineate the pCW3 replication region, a plasmid containing the 9,744-bp ClaI fragment (bp 4628 to 14372) and the *erm*(B) erythromycin resistance gene was constructed. This plasmid, pJIR936, could replicate in *C. perfringens* (K. Koutsis and J. Rood, unpublished data). Subsequently, deletion derivatives of pJIR936 were isolated and tested for their abilities to replicate in *C. perfringens*. The smallest derivative that still supported plasmid replication in this organism was pJIR2768, which carried the *pcw310* to *pcw314* genes (Fig. 3).

To determine which of these genes was essential for plasmid replication, a series of transposon mutants of pJIR2768 was generated by EZ-TN mutagenesis in *Escherichia coli* and mapped. We identified 34 plasmids that had insertions in the pCW3-derived region and independently introduced them into *C. perfringens* strain JIR325. The ability of the plasmid derivatives to replicate was determined by the isolation of erythromycin-resistant transformants and confirmed by plasmid isolation and restriction analysis. None of the derivatives containing an insert within the *pcw314* gene replicated in *C. perfringens* (Fig. 4). These results demonstrated that *pcw314* was essential for plasmid replication, and it was therefore designated the *rep* gene.

The region between *pcw313* and the *rep* gene (Fig. 5) contained five distinct pairs of inverted repeats and four 17-bp direct repeats that could act as iteron-like sequences in the initiation of pCW3 replication. These repeats may represent either iteron-like Rep binding sites or the binding sites for plasmid stability proteins encoded elsewhere in the *rep* region.

TABLE 2. Predicted pCW3 genes

pCW3 gene	Function of closest relative of gene product, source, and/or similarity	Size (aa)	Coding sequence position
tetA(P)	TetA(P) tetracycline efflux resistance protein	420	559-1821
tetB(P)	TetB(P) tetracycline resistance protein	652	1805-3763
pcw303	Hypothetical protein	92	3955-4233
regA	Transcriptional regulator A, AraC related, *Bacillus anthracis* AraC, 150/292 (51%), AraC-type DNA binding domain COG2207	289	4684-5553
pcw305	Conserved hypothetical protein, *C. perfringens* CPE2599, 48/50 (96%)	50	6101-6253
resP	Site-specific serine recombinase-resolvase family, *Bacillus thuringiensis* plasmid pBtoxis, pBt142, 91/210 (43%); *C. perfringens* pIP404, ORF8, 54/150 (36%); *C. perfringens* pCP13, PCP15, 50/150 (33%), resolvase pfam00239\	247	7333-8076
pcw307	Hypothetical protein	218	8195-8851
pcw308	Hypothetical protein	156	9351-9821
regB	Transcriptional regulator B, LexA-like SOS response transcriptional repressor COG1974, *C. perfringens* CPE1161, 68/200 (34%)	176	9888-10418
pcw310	Hypothetical protein	75	10704-10931
pcw311	Conserved hypothetical protein, *C. perfringens* pCP13, PCP12, 76/168 (45%); *C. perfringens* pIP404, ORF6, 49/163 (30%)	165	10997-11494
pcw312	Hypothetical protein	87	11552-11815c
pcw313	Possible plasmid partitioning protein; MreB-like, actin-like ATPase COG1077	308	11794-12720c
rep	Rep, replication protein	276	13239-14069
regC	Transcriptional regulator C, LexA-like SOS response transcriptional repressor COG1974, *C. perfringens* pCP13 PCP61, 135/378 (35%)	344	14336-15370c
regD	Putative transcriptional regulator, *Enterococcus faecalis* EF0306, 19/55 (34%), HTH_XRE cd00093	77	15531-15764
pcw317	Conserved hypothetical protein, *C. perfringens* pCP13 PCP60, 32/119 (26%)	124	15855-16229
pcw318	Conserved hypothetical protein, *C. perfringens* pCP13 PCP59, 184/397 (46%)	366	16290-17390
pcw319	Probable PemK-like plasmid postsegregational toxin, *C. perfringens*, pCP13 PCP58, PemK pfam02452, 73/121 (60%)	124	17458-17832
cna	Probable surface-anchored protein (LPXTG motif), collagen adhesin-like protein, *C. perfringens* pCP13 PCP57, 778/1150 (67%), predicted outer membrane protein COG4932	1,187	17972-21535
pcw321	Hypothetical protein	42	21606-21731
pcw322	Hypothetical protein	85	21750-22007
srtA	Sortase, surface protein transpeptidase, *C. perfringens* pCP13 PCP56, 109/204 (53%), sortase cd0004	214	22017-22661
pcw324	Hypothetical protein	100	22672-22974
pcw325	Hypothetical protein	72	22978-23196
dam	Site-specific DNA methyltransferase (adenine specific), COG0338	254	23260-24024
pcw327	Hypothetical protein	94	24051-24335
pcw328	Hypothetical protein	102	24336-24644
intP	Potential tyrosine recombinase (integrase), pfam00589	266	25036-25836
tcpA	Probable coupling protein with similarity to DNA segregation ATPases FTsK/SpoIIIE and related proteins, COG1674	530	25890-27482
tcpB	Possible DNA segregation ATPase FtsK/SpoIIIE-like ATPase, COG1674, COG0630	327	27497-28480
tcpC	Tn*916* ORF13-like protein, *Enterococcus faecium* Efae2512, 75/305 (24%)	359	28473-29552
tcpD	Hypothetical protein	115	29564-29911
tcpE	Tn*916* ORF17-like protein, *Listeria monocytogenes* lmo1107, 26/93 (27%)	122	29923-30291
tcpF	Tn*916* ORF16-like conjugation protein with predicted ATPase, *Listeria monocytogenes* lmo1106, 226/845 (26%)	841	30349-32874
tcpG	Peptidoglycan hydrolase, *C. perfringens* PCP44, 121/161 (75%), Tn*916* ORF14-like protein, COG1705, FlgJ, muramidase, amidase_4, pfam01832	334	32874-33878
tcpH	Tn*916* ORF15-like membrane protein	832	33981-36479
tcpI	Membrane-bound metal-dependant hydrolase, *Bacillus anthracis* pX02-79, 63/162 (38%)	164	36482-36976
tcpJ	Hypothetical protein	193	36985-37566
pcw340	Hypothetical protein	101	38691-38996
dcm	Site-specific DNA methyltransferase (cytosine specific), pfam00145, *C. perfringens* strain 297442, 153/155 (98%)	339	39011-40030
pcw342	Hypothetical protein	57	40047-40220
pcw343	Conserved hypothetical protein *C. perfringens* strain 297442, 325/340 (95%)	340	40217-41239
pcw344	Hypothetical protein	468	41334-42740
pcw345	Hypothetical protein	133	43328-43729
pcw346	Conserved hypothetical protein *C. perfringens* PCP33, 37/68 (54%)	103	43827-44138
pcw347	Hypothetical protein	77	44450-44683c
pcw348	Hypothetical protein	300	44708-45610c
pcw349	Hypothetical protein	135	45740-46147
pcw350	Conserved hypothetical protein, *C. perfringens* CPE0425, 250/284 (88%)	285	46211-47068
pcw351	Hypothetical protein	29	47085-47174

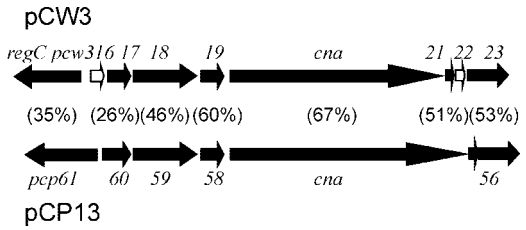

FIG. 2. Genetic comparison of the *cna* locus. Depicted are the arrangement and coding orientation of the genes surrounding the *cna* gene within both pCW3 and pCP13. The percentages of amino acid sequence identities between the encoded proteins are shown in parentheses. Genes present only on pCW3 are filled in with white.

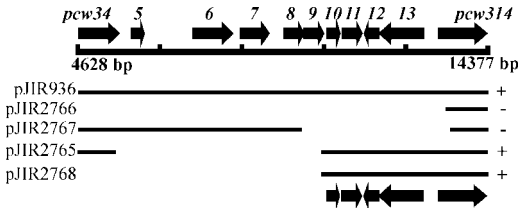

FIG. 3. Localization of the pCW3 plasmid replication region. Shown at the top is the genetic organization of the relevant 9.8-kb ClaI fragment of pCW3. At the bottom, the line diagrams denote the region of pCW3 contained within each of the depicted plasmids. A plus sign indicates that the plasmid replicates independently in *C. perfringens*; a minus sign indicates that the plasmid does not replicate independently in *C. perfringens*.

Several of the EZ-TN inserts were located within this region (Fig. 5), one of which (at bp 13047) was nonfunctional, suggesting that the integrity of at least part of this region is important for either Rep expression or function.

pCW3 encodes functional homologues of Tn*916*-encoded proteins. Several putative proteins encoded within the *pcw329-pcw339* region had varying but low levels of amino acid sequence identity to conjugation proteins encoded by the conjugative transposon Tn*916* (15), although the genetic arrangement was different (Fig. 6). The Tn*916* proteins are involved in conjugative transfer, although their exact functions are not known. Therefore, we postulated that the pCW3 genes were involved in conjugation and designated them the *tcp* genes, for "transfer clostridial plasmid." Bioinformatic analysis suggested that the *intP-tcpJ* genes comprised an operon.

The first gene of the putative *intP-tcpJ* operon encoded a potential site-specific tyrosine recombinase, IntP. Although this protein was somewhat smaller than most tyrosine recombinases, it was similar in size to XerC- and XerD-like recombinases, which are known to be involved in the resolution of chromosome dimers (54). The second gene, *tcpA*, encoded a predicted integral inner membrane protein with two putative N-terminal transmembrane domains and a C-terminal cytoplasmic region that contained consensus Walker A and B boxes. These features are commonly found within coupling proteins such as TraG (RP4), TraD (F plasmid), and TrwB (R388) (22). TcpA also had a conserved domain (COG1674) found within DNA transport proteins, such as FtsK, a DNA segregation ATPase from *E. coli* (43), and SpoIIIE, a protein involved in chromosomal segregation during sporulation of *Bacillus subtilis* (53). Members of the FtsK/SpoIIIE protein family are generally larger than TcpA, and their transmembrane region consists of five membrane-spanning segments (20). Coupling proteins from conjugative plasmids often share functional and structural features with members of the FtsK/SpoIIIE protein family. TcpA and ORF21 from Tn*916* had very little amino acid sequence identity but may have similar functions, since they both have similarity to members of this family. The next pCW3 gene, *tcpB*, encoded a predicted cytoplasmic protein that had 28% amino acid sequence identity to the central section of TcpA and a less well-conserved FtsK/SpoIIIE domain.

tcpC was one of five genes whose products had low-level sequence identity to Tn*916* proteins, having similarity to ORF13 homologues from Tn*916*, Tn*5397*, and CW459*tet*(M). The other putative pCW3 products that had similarity to Tn*916* homologues included TcpE (ORF17), the potential ATPase TcpF (ORF16), a putative peptidoglycan hydrolase, TcpG (ORF14), and TcpH (ORF15). Very little is known about the function of these proteins in Tn*916*.

TcpH was predicted to be an integral membrane protein, with eight potential transmembrane domains in the first half of the protein. Within this section of TcpH and ORF15 was a region that had similarity to a conserved TrbL/VirB6 domain. VirB6 from *Agrobacterium tumefaciens* is an integral membrane protein that is involved in mating pair formation (Mpf), in particular in the stabilization of other Mpf-related proteins (25). It is possible that TcpH may play a role similar to that of VirB6, but it may have additional functions, since it has a cytoplasmic region of 402 amino acids that is not found within VirB6.

TcpG appeared to have an N-terminal signal peptide as well as both the N-terminal catalytic domain and the C-terminal cell wall binding domains common to peptidoglycan hydrolases, in particular to *N*-acetylmuramoyl-L-alanine amidases. TcpG was similar to PCP44 from pCP13, with similarity limited to the predicted cell wall binding region of the protein. TcpG did not contain the same putative amidase domain as PCP44. TcpI was another potential inner membrane protein, with three predicted transmembrane domains. It was most similar to pX02-79, a conserved hypothetical protein encoded by the plasmid pX02 from *Bacillus anthracis*, and also contained a predicted membrane-bound metal-dependent hydrolase domain. We suggest that TcpG and TcpI may work synergistically to digest the cell wall so that conjugative transfer can occur.

All known conjugative plasmids encode a relaxase protein that contains a highly conserved motif. However, we were unable to locate a pCW3-encoded protein that contains this motif. Similarly, we were not able to identify a potential origin of transfer within the pCW3 sequence.

Allelic exchange was used to determine if Tn*916* homologues located within the *tcp* region were required for conjugative transfer. The *tcpF* and *tcpH* genes were chosen as targets, because TcpF was the protein most similar to its Tn*916* homologue and because TcpH was a putative inner membrane Mpf protein. Gene regions (ca. 2 kb) flanking either *tcpF* or *tcpH* were cloned on either side of the *erm*(Q) erythromycin resistance gene on the suicide vector pJIR2715 (C. Hennequin, K. Farrow, and J. Rood, unpublished data). Elsewhere on this

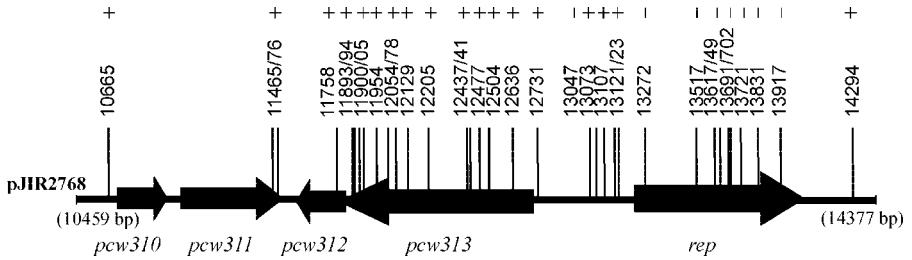

FIG. 4. Identification of the *rep* gene. The arrows indicate the pCW3-derived genes within the cloned insert of pJIR2768. Vertical lines with numbers represent independent EZ::TN insertion mutants within pJIR2768. Insertion derivatives are named by corresponding base pairs within the pCW3 sequence. A plus sign indicates that the EZ::TN-containing pJIR2768 derivative plasmid replicates independently in *C. perfringens*; a minus sign indicates that the EZ::TN-containing pJIR2768 derivative plasmid does not replicate independently in *C. perfringens*.

vector, there is a chloramphenicol resistance gene (*catP*) that enables screening for double crossovers by selecting colonies that are susceptible to chloramphenicol or thiamphenicol. The suicide plasmids pJIR3024 (*tcpF*) and pJIR2899 (*tcpH*) were constructed and used to transform *C. perfringens* strain JIR325(pCW3) to erythromycin resistance. Two independently derived mutants of each of the *tcpF* and *tcpH* genes were isolated, and PCR analysis was used to confirm that they were derived from double crossovers onto pCW3 (data not shown).

The resultant pCW3Δ*tcpF*::*erm*(Q) or pCW3Δ*tcpH*::*erm*(Q) mutant was unable to encode conjugative transfer (Table 3). To establish that the loss of conjugative transfer was the result of the specific mutation, complementation analysis was performed. The wild-type *tcpF* and *tcpH* genes were cloned independently into the *C. perfringens-E. coli* shuttle vector pJIR750, and the resultant plasmids were introduced into strains harboring the mutated pCW3 plasmids. Conjugation experiments showed that complementation in *trans* restored conjugative ability to both the *tcpF* and *tcpH* mutants (Table 3), although not to wild-type levels. This result may be due to polar effects on the genes located downstream of *tcpF* and *tcpH* on the mutated plasmids or different expression levels of the genes located on the shuttle plasmids. Nonetheless, it is clear that both mutants can be complemented in *trans* and therefore that these genes are essential for conjugative transfer of pCW3, which confirms experimentally that genes within the *tcp* region are involved in conjugative transfer.

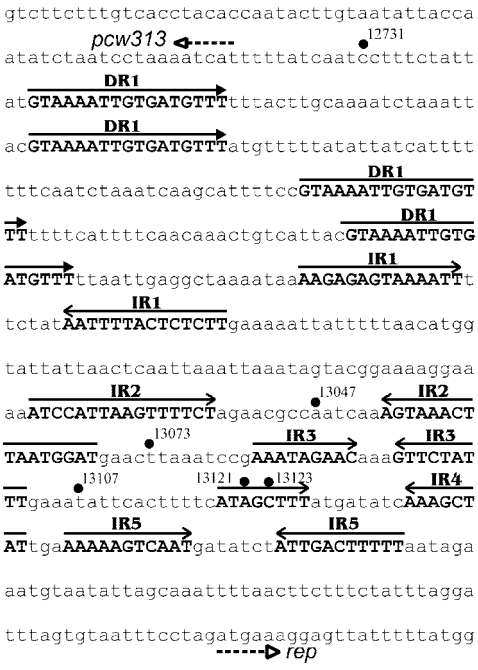

FIG. 5. Repeated elements in the intergenic region between the *pcw313* and *rep* genes. Depicted is the pCW3 nucleotide sequence from bp 12661 to 13260. Closed arrowheads denote the four copies of the identical direct repeats (DR1). The open-headed arrows depict the five pairs of perfect inverted repeats (IR1 to IR5). Shaded circles highlight insertion sites of EZ::TN derivatives in this region. The dotted line shows the location of the *pcw313* and *rep* genes.

All known conjugative plasmids from *C. perfringens* carry the *tcp* genes. Since the area of identity between pCW3 and other conjugative *C. perfringens* plasmids appeared to encompass the *tcp* region, we used PCR analysis with 10 overlapping primer pairs to show that 11 diverse strains that harbored conjugative tetracycline resistance plasmids had the *tcpEFGH* gene region. More extensive analysis was carried out on the conjugative tetracycline resistance plasmid, pJIR26 (the plasmid most different from pCW3), and pMRS4969, a genetically marked derivative of the conjugative *cpe* plasmid pCPF4969 (12). PCR amplification across the entire *intP-dcm* region was performed, and the resultant products were completely sequenced. The *tcp* regions of all three conjugative plasmids were very similar (Fig. 7), with the homologous proteins having 88% to 99.4% amino acid sequence identity. The exceptions were the TcpA proteins, which all had the same FtsK/SpoIIIE-like domains but only had 54% to 61% identity, and TcpJ, which in pCW3 was truncated by approximately 70 amino acids. In addition, there was no *tcpB* gene on pJIR26. Upstream of *tcpC*

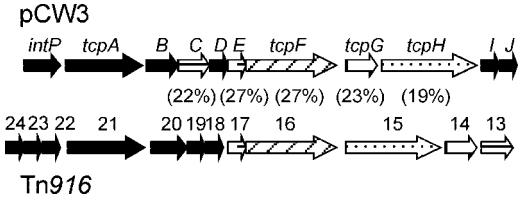

FIG. 6. Genetic comparison of the transfer-related regions of pCW3 and Tn*916*. Numbers in parentheses denote the percentages of amino acid sequence identity between the encoded proteins. Related genes are shaded in a similar manner. Genes shaded black encode proteins with no similarity.

TABLE 3. Conjugation frequencies of *tcpF* and *tcpH* mutants and their complemented derivatives

Plasmid	Conjugation frequency (transconjugants/donor cell)
pCW3	$(1.4 \pm 0.1) \times 10^{-1}$
pCW3 Δ*tcpH1*::*erm*(Q)	$<(1.1 \pm 0.2) \times 10^{-8}$
pCW3 Δ *tcpH2*::*erm*(Q)	$<(1.3 \pm 0.2) \times 10^{-8}$
pCW3 Δ*tcpH1*::*erm*(Q)(p*tcpH*$^+$)	$(1.2 \pm 0.2) \times 10^{-4}$
pCW3 Δ*tcpH2*::*erm*(Q)(p*tcpH*$^+$)	$(4.8 \pm 2.2) \times 10^{-5}$
pCW3 Δ*tcpF1*::*erm*(Q)	$<(8.1 \pm 1.1) \times 10^{-9}$
pCW3 Δ*tcpF2*::*erm*(Q)	$<(1.8 \pm 0.2) \times 10^{-8}$
pCW3 Δ*tcpF1*::*erm*(Q)(p*tcpF*$^+$)	$(1.3 \pm 0.6) \times 10^{-6}$
pCW3 Δ*tcpF2*::*erm*(Q)(p*tcpF*$^+$)	$(2.7 \pm 0.6) \times 10^{-6}$

in pJIR26, there was an ORF also found in pMRS4969. pJIR26 also had an additional ORF between *tcpE* and *tcpF*.

Compared to pCW3, pMRS4969 contained two large insertions and a deletion. The first insertion introduced three additional ORFs between *tcpA* and *tcpB*, the largest encoding an additional FtsK-like protein, ORF204, that had 31 to 39% identity to the TcpA and TcpB proteins of pCW3. The other ORFs encoded two small hypothetical proteins. The second insertion consisted of a group II intron, C.p.In1A, that was located within the 3′ end of *tcpF*. The putative reverse transcriptase of C.p.In1A had 55 to 60% similarity to enzymes from group II introns of *Enterococcus* and *Bacillus* spp. and 43% identity to the reverse transcriptase of the group II intron located on Tn*5397*. The deletion within pMRS4969 removed the gene encoding the small hypothetical protein PCW340, located upstream of the *dcm* gene.

Finally, the genes for many of the major lethal toxins produced by *C. perfringens* are known to be carried on large plasmids (42). Sequence analysis of some of these plasmids is under way as part of a collaborative project involving several laboratories. Analysis of data made available from this project (G. Myers, I. Paulsen, J. Songer, B. McClane, R. Titball, J. Rood, and S. Melville, personal communication) indicated that a plasmid carrying the β-toxin gene from a *C. perfringens* type C strain and a separate plasmid carrying the ε-toxin gene from a *C. perfringens* type D strain contained transfer regions to almost identical those found within pCW3 (Fig. 7). The type C plasmid was missing *tcpB* and had a small ORF also present in pJIR26. The type D plasmid had *tcpB* and two group II introns, one of which (C.p.In1B) had a homologue on pMRS4969. Also

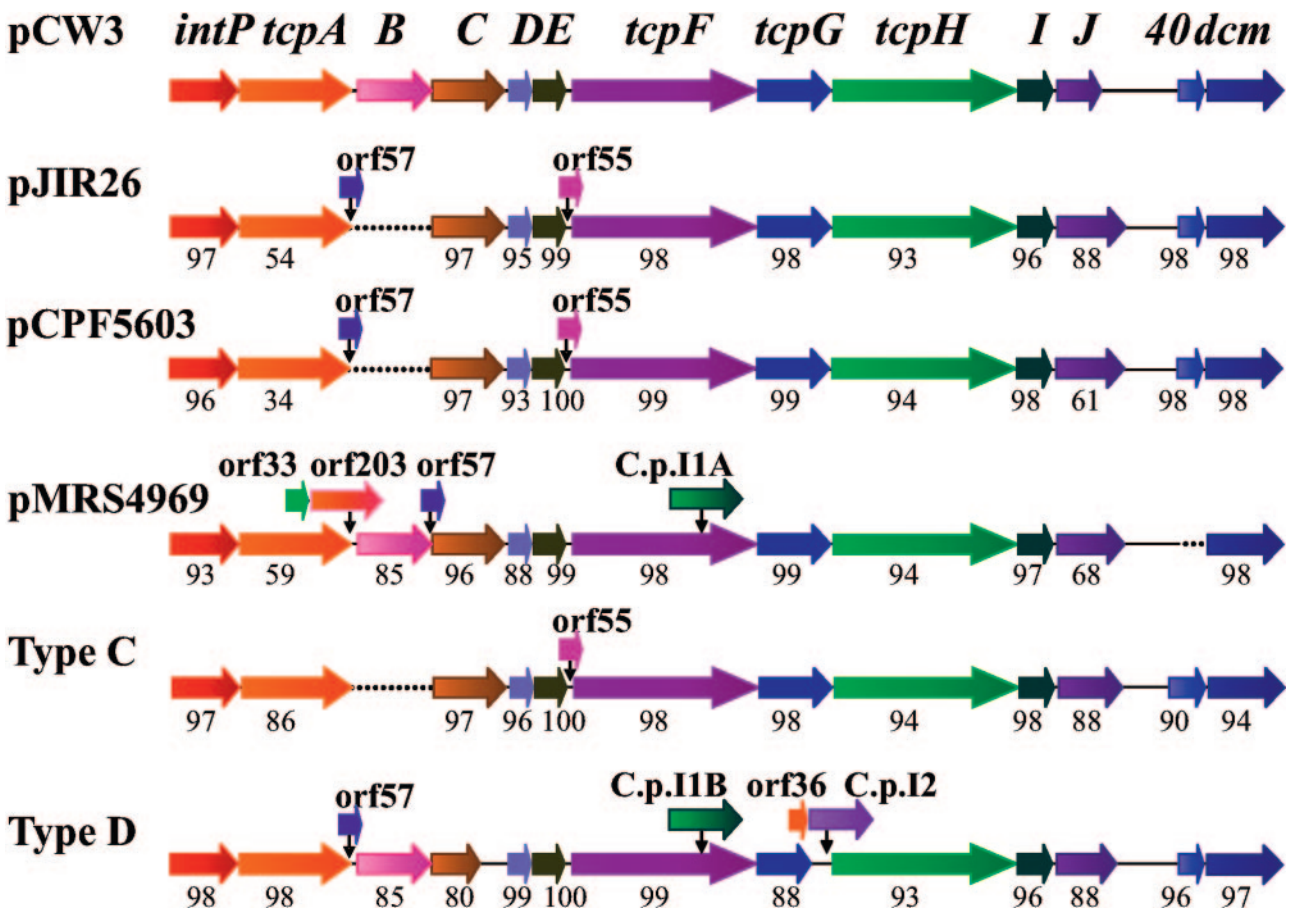

FIG. 7. Comparative analysis of the *intP* to *dcm* region. The genetic organization of this region within pCW3 is depicted at the top of the diagram. The genetic maps of the *tcp* regions of the tetracycline resistance plasmid pJIR26 and the CPE-derived plasmid pMRS4969 were determined as part of this study, and that of pCPF5603 is from Miyamoto et al. (36). The comparative maps of the equivalent regions from type C and type D toxin plasmids were derived from data from The Institute for Genomic Research (see the text). Insertions with respect to the pCW3 sequence are indicated by arrows above the linear maps. Deletions with respect to the pCW3 sequence are depicted by dotted lines. Related genes are shaded in a similar manner. Percentages indicate amino acid sequence identity to the equivalent pCW3 homologue.

included in this comparison was the *tcp* region from another *cpe* plasmid, pCPF5603, which has very recently been reported (36). The *tcp* region of this plasmid, which has not yet been shown to be conjugative, has the same genetic organization as the conjugative tetracycline resistance plasmid pJIR26 (Fig. 7).

DISCUSSION

In this study, we have shown that the *tcp* conjugation region from pCW3 is present in all known conjugative plasmids from *C. perfringens*, including a plasmid that encodes the enterotoxin. It is also present on plasmids encoding β-toxin or ε-toxin, which are major lethal toxins produced by *C. perfringens.* Based on our functional data, we suggest that the β-toxin and ε-toxin plasmids may also be conjugative. This finding has significant implications for the epidemiology of diseases caused by these strains. As previously discussed in relation to the enterotoxin plasmid (12), it may no longer be necessary for an invading *C. perfringens* isolate to have the ability to colonize the gastrointestinal tract. By conjugative transfer of a β-toxin and ε-toxin plasmid to an already adherent *C. perfringens* cell, the resident bacterium could acquire the potential to produce the relevant toxin and thereby cause disease.

Very recently the complete sequences of the *cpe* plasmids pCPF5603 and pCPF4969 from *C. perfringens* were reported (36). Both plasmids had the *tcp* region, and PCR analysis indicated that this region was present in other *cpe* plasmids and plasmids from type B to E strains of *C. perfringens*. The sequence analysis of the *tcp* region from pCPF4969 is in agreement with the sequence of its derivative, pMRS4969, reported here. The demonstration in the current study that genes located in the common *tcp* region were essential for conjugative transfer provides experimental evidence to support the hypothesis that some or all of the toxin plasmids from *C. perfringens* are conjugative.

Comparative genomic analysis indicated that the products of the *tcp* transfer region had limited but significant similarity to conjugation proteins from Tn*916*. Transposon mutagenesis of Tn*916* has identified a region carrying 12 genes (*orf24-orf13*) that is involved in the conjugative transfer process, including the respective Tn*916* homologues of TcpF and TcpH, ORF15 and ORF16 (13, 16). These genes are present in all functional members of the Tn*916* family, including Tn*5397* from *C. difficile*, although to our knowledge no studies confirming the functional role of individual proteins within these elements have been reported. Since only five of these genes (*orf13* to *orf17*) encode products with similarity to the pCW3 conjugation proteins, it is clear that the conjugative transfer mechanisms of pCW3 and Tn*916*, although related, must involve distinct processes.

Many of the conjugative plasmids identified from gram-positive bacteria have significant similarity (16). These plasmids include the staphylococcal plasmids pG01 and pSK41 (37) (10), the lactococcal plasmid pMRC01 (18), the enterococcal plasmid pRE25 (51), and the streptococcal plasmid pIP501 (27). The proteins encoded by the shared 11-kb transfer region have 80% to 100% identity, suggesting that these plasmids utilize a common conjugation mechanism. However, the conjugation proteins from these plasmids have very little primary sequence similarity to the proteins encoded by conjugative transposons or pCW3.

A second group of conjugative plasmids from gram positives includes the closely related enterococcal plasmids pAD1 and pAM373. Transposon mutagenesis of pAD1 was used to identify a contiguous region of 31 kb that contained genes involved in conjugation (19), and more recently its complete sequence was determined (21). Although the pAD1 conjugation machinery appears to be more complex in terms of the number of proteins involved, it does include homologs of the ORF15 and ORF16 proteins of Tn*916* (17). Genes similar to the transfer genes of pAD1, including *orf15* and *orf16*, are also present on the conjugative plasmid pAM373 and on pTEF1, a plasmid from *E. faecalis* strain V583 (41). However, none of these genes have been reported to be functional conjugation genes.

Deletion and mutagenesis studies have led to the identification of the pCW3 *rep* gene, which was shown to be essential for plasmid replication. Note that the putative Rep protein had a basic pI of 10, as expected for a DNA binding protein, but it had no significant similarity to any known Rep initiator proteins from other plasmids. However, a protein with 98% identity to Rep is encoded by both pCPF5603 (pCPF5603_16) and pCPF4969 (pCPF4969_01), although its function was not previously recognized (36). We suggest that these toxin plasmids replicate by a mechanism similar to that of pCW3 and that Rep is a very *C. perfringens*-specific protein, which would explain why homologues of pCW3 or the toxin plasmids have not been found in any other bacterial species.

In conclusion, the derivation of the complete sequence of pCW3 from *C. perfringens* and the functional identification of the conjugation and replication regions, coupled with the comparative analysis of the equivalent regions of several other conjugative plasmids from *C. perfringens*, have identified a unique family of conjugative plasmids that to date are restricted to this species. These closely related plasmids carry either a novel tetracycline resistance operon that has not been found in any other genus or toxin genes that are restricted to *C. perfringens*. These plasmids presumably evolved in *C. perfringens* from a common progenitor. The presence of an integrase gene, the first gene in the putative *tcp* operon, and the fact that the products of the conjugation genes within this novel plasmid family have similarity to conjugation proteins encoded by Tn*916* support the hypothesis that the *tcp* region evolved from an exogenous precursor, presumably from the conjugative transfer of a Tn*916*-like ICE into *C. perfringens*, followed by its insertion into a nonconjugative native plasmid. Subsequently, evolution of this now-conjugative plasmid has led to the loss of the ability of the ICE to excise independently, the rearrangement and divergent development of the conjugation genes, and the acquisition of different genes that confer a selective advantage to the host, such as toxin and antibiotic resistance genes. This hypothesis is supported by previous studies that showed that Tn*916* is able to integrate into the *C. perfringens* genome after conjugative transfer (8, 26, 33), evidence that Tn*916*-like *tet*(M) genes are relatively common in *C. perfringens* (31), and the fact that a defective element closely related to Tn*916* is present in at least one nonconjugative isolate of *C. perfringens* (46). Finally, we propose that pCW3 may represent an evolutionary intermediate between conjugative plasmids that replicate independently and nonreplicating

ICEs such as Tn*916*. However, the various recombination events that led to the evolution of pCW3 do not appear to have been recent, since this plasmid is only distantly related to Tn*916*.

ACKNOWLEDGMENTS

We thank Torsten Seemann from the Victorian Bioinformatics Consortium for his most valuable assistance.

W.L.T. was the recipient of a Monash Postgraduate Scholarship. This research was supported by grants from the Australian Research Council to the ARC Centre of Excellence in Structural and Functional Microbial Genomics and grant AI056177-03 from the United States National Institute of Allergy and Infectious Diseases.

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