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TcpA, an FtsK/SpoIIIE Homolog, Is Essential for Transfer of the Conjugative Plasmid pCW3 in *Clostridium perfringens*▽†

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The conjugative tetracycline resistance plasmid pCW3 is the paradigm conjugative plasmid in the anaerobic gram-positive pathogen *Clostridium perfringens*. Two closely related FtsK/SpoIIIE homologs, TcpA and TcpB, are encoded on pCW3, which is significant since FtsK domains are found in coupling proteins of gram-negative conjugation systems. To develop an understanding of the mechanism of conjugative transfer in *C. perfringens*, we determined the role of these proteins in the conjugation process. Mutation and complementation analysis was used to show that the *tcpA* gene was essential for the conjugative transfer of pCW3 and that the *tcpB* gene was not required for transfer. Furthermore, complementation of a pCW3Δ*tcpA* mutant with divergent *tcpA* homologs provided experimental evidence that all of the known conjugative plasmids from *C. perfringens* use a similar transfer mechanism. Functional genetic analysis of the TcpA protein established the essential role in conjugative transfer of its Walker A and Walker B ATP-binding motifs and its FtsK-like RAAG motif. It is postulated that TcpA is the essential DNA translocase or coupling protein encoded by pCW3 and as such represents a key component of the unique conjugation process in *C. perfringens*.

Clostridium perfringens is the causative agent of several important histotoxic and enterotoxic diseases of humans and animals (48, 58, 62). Integral to the virulence of *C. perfringens* is its large repertoire of toxins, several of which are encoded on plasmids that appear to be conjugative (8, 11, 33, 40, 52). Extensive restriction endonuclease analysis of conjugative tetracycline resistance plasmids from *C. perfringens* has shown that the 47-kb plasmid pCW3 is the prototype conjugative plasmid in this bacterium (2, 3). Analysis of pCW3 previously focused on the inducible *tet*(P) operon, which confers tetracycline resistance (1, 27, 57). More recent studies have involved determination of the complete sequence of pCW3 and identification of its unique replication protein (8).

Analysis of the pCW3 sequence identified a locus that encodes several gene products with low-level similarity to conjugation proteins from the conjugative transposon Tn*916* (8). This region was designated the *t*ransfer *c*lostridial *p*lasmid (*tcp*) locus and is required for transfer, as shown by the isolation of independent *tcpF* and *tcpH* mutants and subsequent complementation studies (8). Since the region that encompasses the *tcp* locus is conserved in all conjugative plasmids from *C. perfringens* (2, 3, 8, 11, 40), it is likely that the conjugative transfer of both antibiotic resistance and toxin plasmids from this bacterium utilizes a common mechanism.

The mechanism of conjugative transfer between gram-negative cells has been studied extensively (13, 32). In this process the movement of the transferred DNA from DNA-processing proteins, such as the relaxase, to the export proteins that make up the mating pair formation (MPF) complex is facilitated by a coupling protein. Although this precise mechanism has yet to be demonstrated with conjugative plasmids from gram-positive bacteria, bioinformatic analysis has identified considerable similarity between proteins encoded by these plasmids and conjugation systems from gram-negative bacteria (23). Significant similarity has been observed between conjugative plasmids, such as the streptococcal plasmid pIP501 (25, 31), the staphylococcal plasmid pSK41 (18), the lactococcal plasmid pMRC01 (15), and pheromone-induced plasmids from enterococci, such as pAD1 and pAM373 (19). This similarity has allowed identification of key conjugation proteins, such as putative mating channel proteins, relaxases, and coupling proteins, and therefore has led to the hypothesis that gram-positive conjugation systems utilize a mechanism similar to that of their counterparts in gram-negative bacteria (23).

Coupling proteins have two N-terminal transmembrane domains (TMDs) and a C-terminal cytoplasmic region that contains consensus Walker A and B ATP-binding sites (20). These sites are located within conserved VirD4 (COG3505), TraG (pfam02534), and TrwB (cd01127) domains. The cytoplasmic domain of TrwB (TrwBΔN70), the coupling protein from R388, has been crystallized, and its structure has been elucidated. Like F_1-ATPases, it is a homohexameric protein complex with a large (20-Å) central channel, through which single-stranded DNA (ssDNA) may be pumped during conjugative transfer (22).

Coupling proteins belong to the same superfamily as DNA translocases such as FtsK and SpoIIIE. FtsK is a bifunctional protein in which the N-terminal domain is involved in cell division and the C-terminal domain is essential for correct chromosomal segregation (34, 65). To modulate chromosomal segregation, FtsK forms a ring-shaped multimeric DNA-binding complex that uses its ATPase activity to move along the double-stranded DNA (dsDNA) (9, 16). SpoIIIE is a DNA

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export protein that acts as a dsDNA pump to transfer DNA from the mother cell to the forespore during sporulation in *Bacillus subtilis* (56). FtsK-like DNA translocases are large proteins (800 to 1,200 amino acids [aa]) with five N-terminal TMDs and three conserved regions associated with ATP binding and hydrolysis, namely, the Walker A and B ATP-binding motifs (63) and an RAAG motif (gR-GxhLxxatQ) (16). FtsK-like DNA translocases differ from coupling proteins, all of which have an α-helical domain (AAD) inserted between the Walker A and Walker B motifs. Despite these differences, the structure of these proteins includes a common fold, and FtsK-like DNA translocases and coupling proteins may have similar mechanisms of action (16).

Bioinformatic analysis of pCW3 identified two potential proteins, TcpA and TcpB, that have FtsK-like domains (8). These putative DNA translocases may be involved in the movement of DNA and therefore may perform a role similar to that of the coupling proteins in other conjugation systems. TcpA was predicted to be an integral inner membrane protein with an N-terminal region containing two putative TMDs and a C-terminal cytoplasmic region containing a conserved FtsK/SpoIIIE domain (8). The FtsK/SpoIIIE domain of TcpA encompasses the Walker A and Walker B motifs, as well as the RAAG motif. TcpB, a predicted cytoplasmic protein, also carries the FtsK/SpoIIIE domain identified in TcpA, although it is less well conserved. Therefore, TcpB also has the potential to act as a DNA translocase during the conjugative transfer of pCW3.

To investigate the role of the *tcpA* and *tcpB* genes in the conjugative transfer of pCW3, we isolated a pCW3Δ*tcpAB* mutant that was shown to be conjugation deficient and by performing complementation studies demonstrated that *tcpA* was essential for conjugative transfer. A pCW3Δ*tcpA* mutant was also constructed and used to confirm the relationship between various conjugative plasmids from *C. perfringens* by demonstrating the functional interchangeability of highly divergent *tcpA* homologs in complementation studies. Functional genetic studies of the conserved TcpA domains revealed that the FtsK-like motifs were essential for the function of TcpA and the conjugative transfer of pCW3.

MATERIALS AND METHODS

Bacterial strains and plasmids. The *C. perfringens* strains used in this study are described in Table 1 and were cultured at 37°C in TPG broth (49), brain heart infusion broth (Oxoid), FTG medium (Difco), or nutrient agar (46) supplemented with tetracycline (10 μg/ml), rifampin (10 μg/ml), nalidixic acid (10 μg/ml), thiamphenicol (10 μg/ml), or streptomycin (200 μg/ml), when needed. When required, 1% (vol/vol) saturated potassium chlorate was included. *C. perfringens* agar cultures were incubated in an atmosphere containing 10% H_2, 10% CO_2, and 80% N_2. The *Escherichia coli* host strain used was DH5α (Life Technologies), which was grown at 37°C in 2×YT medium (39) supplemented with erythromycin (150 μg/ml) or chloramphenicol (30 μg/ml). Plasmids are also listed in Table 1.

Molecular techniques. *E. coli* plasmid DNA was isolated by an alkaline lysis method performed according to the manufacturer's instructions (QIAGEN). Purified *C. perfringens* DNA was obtained as described previously (47). For PCR amplification *Taq* DNA polymerase (Roche) and 0.5 μM of each primer were used. Denaturation (94°C for 30 s), annealing (50°C for 30 s), and extension (72°C for 3 to 5 min) steps were carried out for 30 cycles. Sequence analysis of constructs was performed with an Applied Biosystems 3730S capillary sequencer. Sequence data were analyzed using Vector NTI (Invitrogen) in conjunction with the Sanger Institute freeware Artemis, release 6. TcpA sequences were aligned using ClustalW (61) and T Coffee (42). Details concerning oligonucleotides are shown in Table S1 in the supplemental material.

Construction of *C. perfringens* mutants by allelic exchange. *C. perfringens* suicide vectors contained ca. 2 kb of sequence upstream and downstream of the gene to be mutated. These regions were generated by PCR and cloned into the pT7Blue cloning vector (Novagen). Each product was then subcloned sequentially into the *E. coli* vector pJIR2715 (8) (Table 1). This vector, which replicates in *E. coli* but not in *C. perfringens*, encodes chloramphenicol (or thiamphenicol) and erythromycin resistance. To isolate a derivative of pCW3 from which both *tcpA* and *tcpB* were deleted, a Δ*tcpAB* suicide vector was constructed as follows. The bp 23973 to 25913 pCW3 region upstream of *tcpA* was cloned upstream of *erm*(Q), and the bp 28420 to 30572 region downstream of *tcpB* was cloned downstream of *erm*(Q) to generate the suicide vector pJIR3099. Similarly, a Δ*tcpA* suicide vector was constructed by cloning the bp 23973 to 25913 and bp 27476 to 29767 regions of pCW3 into pJIR2715 upstream and downstream, respectively, of *erm*(Q) to form pJIR3211. The suicide vectors were independently introduced into JIR4195 [i.e., JIR325(pCW3)] by electroporation (55). In these experiments, transformation of these suicide vectors into *C. perfringens* and selection for erythromycin resistance selected for strains in which the plasmid had integrated into the host genome, presumably within pCW3. Screening for thiamphenicol sensitivity identified putative mutants derived from double-crossover events. DNA was purified from potential erythromycin-resistant thiamphenicol-sensitive recombinants, and PCR and Southern blotting (59) were used to confirm the replacement of the target gene(s) with the *erm*(Q) cassette and loss of the suicide plasmid.

Construction of complementation vectors. PCR products carrying the pCW3-derived wild-type *tcpA*, *tcpB*, and *tcpAB* gene regions were generated using the primer pairs JRP2244/JRP2274, JRP1370/JRP2356, and JRP2244/JRP2356, respectively, and were cloned into the *C. perfringens-E. coli* shuttle vector pJIR750 via pT7Blue, generating pJIR3212, pJIR3105, and pJIR3209, respectively. The *tcpA* gene was also amplified from *C. perfringens* plasmids pJGS1495, pJIR26, and pMRS4969, which is a derivative of pCPF4969 (50). Due to sequence differences the same 5′ primer but different 3′ primers were used. The resulting PCR products were cloned directly into pJIR750 to generate pJIR3333, pJIR3334, and pJIR3335, respectively. Various site-directed mutants or internal deletions of the *tcpA* gene were isolated using a QuikChange mutagenesis kit (Stratagene). The starting vector in these experiments was a pT7Blue*tcpA*$^+$ derivative, pJIR3096. Oligonucleotide pairs JRP3406/JRP3407, JRP3408/JRP3409, and JRP3410/JRP3411 were used to construct the *tcpA* mutants K242A, DE334/335AA, and Q379A, respectively. Plasmids encoding the TMD deletion derivatives $TcpA_{\Delta 46\text{-}69}$, $TcpA_{\Delta 79\text{-}104}$, and $TcpA_{\Delta 46\text{-}104}$ were also generated by QuickChange mutagenesis of pJIR3096 using oligonucleotide pairs JRP2827/JRP2828, JRP2829/JRP2830, and JRP2831/JRP2832, respectively. Each pJIR3096 derivative was then subcloned into pJIR750 to generate the mutated complementation vectors pJIR3340, pJIR3342, and pJIR3344, as well as the TMD deletion complementation vectors pJIR3346, pJIR3348, and pJIR3350, respectively. *tcpA* derivatives encoding $TcpA_{1\text{-}469}$, $TcpA_{1\text{-}365}$, and $TcpA_{1\text{-}316}$ were constructed using PCR products amplified using JRP2244 and reverse primers JRP2645, JRP2824, and JRP2825, respectively. Each *C. perfringens* strain that contained one of these complementation vectors was confirmed by restriction endonuclease analysis of PCR products and sequence analysis where appropriate.

Conjugations. Matings on solid media were carried out as described previously (46, 47). Nutrient agar supplemented with tetracycline, streptomycin, and potassium chlorate was used to select for transconjugants when strain JIR4394 was used as the recipient. The efficiency of conjugative transfer was expressed as the number of transconjugants per donor cell.

RESULTS

TcpA protein is a member of the FtsK superclade. Bioinformatic analysis of the putative *tcpA* gene product identified ATP-binding Walker boxes A and B within an FtsK-like conserved domain (COG1674) and a similar, although less well conserved, domain within TcpB (8). FtsK domains are also present within coupling proteins, but further analysis of the 530-aa TcpA protein did not identify any domains specific to coupling proteins. To investigate the relationship between TcpA, TcpB, DNA translocases, and coupling proteins, their amino acid sequences were aligned (data not shown) and their putative domain structures were compared (Fig. 1A). The results indicated that TcpA shares similar domain features with FtsK and several coupling proteins.

TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Description[a]	Reference(s) or source
C. perfringens strains		
JIR325	Strain 13 derivative, Rif^r Nal^r	36
JIR4195	JIR325(pCW3) Rif^r Nal^r Tc^r	P. Johanesen, D. Lyras, and J. Rood, unpublished
JIR4394	Strain 13 derivative, Sm^r Chl^r	8
JIR4974	JIR325(pJIR3101) Rif^r Nal^r Tc^r Em^r	This study
JIR4975	JIR325(pJIR3102) Rif^r Nal^r Tc^r Em^r	This study
JIR12051	JIR325(pJIR3213) Rif^r Nal^r Tc^r Em^r	This study
JIR12052	JIR325(pJIR3214) Rif^r Nal^r Tc^r Em^r	This study
Plasmids		
pT7Blue	*E. coli* cloning vector, bla^+, f1 origin, pUC origin, *lacZ* α-peptide	Novagen
pCW3	Confers conjugative tetracycline resistance	8, 49
pCPF4969	CPE toxin plasmid from F4969	40
pMRS4969	pCPF4969*cpe*::*catP*	50
pJGS1495	β-Toxin plasmid from JGS1495	J. G. Songer (University of Arizona)
pJGS1721	ε-Toxin plasmid from JGS1721	J. G. Songer (University of Arizona)
pJIR26	pJIR27ΔTn*4452*	3
pJIR750	*E. coli-C. perfringens* shuttle vector, $catP^+$ *lacZ* α-peptide	7
pJIR1909	pCW3 10,358-bp ClaI (bp 41533 to 4628) plus pCW3 9,744-bp ClaI (bp 4628 to 14372)	8
pJIR2715	Base plasmid for construction of *C. perfringens* suicide vectors, *erm*$(Q)^+$ $catP^+$ $oriT^+$	8
pJIR2774	Conjugative lincomycin resistance plasmid	D. Lyras, J.G. Songer, and J. Rood, unpublished
pJIR3093	pT7Blue (EcoRV) ΩJRP2025/JRP2026 PCR product (1,940 bp) (upstream of *tcpA*)	This study
pJIR3094	pT7Blue (EcoRV) ΩJRP2027/JRP2028 PCR product (2,291 bp) (downstream of *tcpA*)	This study
pJIR3095	pT7Blue (EcoRV) ΩJRP2029/JRP2030 PCR product (2,152 bp) (downstream of *tcpB*)	This study
pJIR3096	pT7Blue(EcoRV) ΩJRP2044/JRP2074 PCR product (2,126 bp) (upstream of *tcpB*, $tcpA^+$)	This study
pJIR3097	pJIR2715 (XhoI/SacI) ΩpJIR3095 (XhoI/SacI; 1,940 bp) (3′ *tcpAB* suicide vector)	This study
pJIR3099	pJIR3097 (SphI/BamHI) ΩpJIR3095 (SphI/BamHI; 2,152 bp) (*tcpAB* suicide vector)	This study
pJIR3101	pCW3Δ*tcpAB1*	Transformation with pJIR3099
pJIR3102	pCW3Δ*tcpAB2*	Transformation with pJIR3099
pJIR3104	pT7Blue (EcoRV) ΩJRP1370/JRP2356 PCR product (1,659 bp) ($tcpB^+$)	This study
pJIR3105	pJIR750 (SphI/BamHI) ΩpJIR3104 (SphI/BamHI; 1,659 bp) ($tcpB^+$ complementation vector)	This study
pJIR3207	pT7Blue (EcoRV) ΩJRP2244/JRP2356 PCR product (3,145 bp) ($tcpA^+B^+$)	This study
pJIR3209	pJIR750 (SphI/BamHI) ΩpJIR3207 (SphI/BamHI; 3,145 bp) ($tcpA^+B^+$ complementation vector)	This study
pJIR3211	pJIR3099 (SphI/BamHI) ΩpJIR3094 (SphI/BamHI; 2,291 bp) (*tcpA* suicide vector)	This study
pJIR3212	pJIR750 (SphI/BamHI) ΩJRP2244/JRP2274 PCR product (SphI/BamHI; 2,126 bp) ($tcpA^+$ complementation vector)	This study
pJIR3213	pCW3Δ*tcpA1*	Transformation with pJIR3211
pJIR3214	pCW3Δ*tcpA2*	Transformation with pJIR3211
pJIR3333	pJIR750 (SphI/BamHI) ΩJRP2244/JRP3433 PCR product (SphI/BamHI; 2,126 bp) ($tcpA_{pJGS1495}$ complementation vector)	This study
pJIR3334	pJIR750 (SphI/BamHI) ΩJRP2244/JRP3433 PCR product (SphI/BamHI; 2,126 bp) ($tcpA_{pJIR26}$ complementation vector)	This study
pJIR3335	pJIR750 (SphI/BamHI) ΩJRP2244/JRP3432 PCR product (SphI/BamHI; 2,126 bp) ($tcpA_{pCPF4969}$ complementation vector)	This study
pJIR3336	pJIR750 (SphI/BamHI) ΩJRP2244/JRP2645 PCR product (SphI/BamHI; 1,861 bp) ($tcpA_{1\text{-}469}$ complementation vector)	This study
pJIR3337	pJIR750 (SphI/BamHI) ΩJRP2244/JRP2824 PCR product (SphI/BamHI; 1,546 bp) ($tcpA_{1\text{-}365}$ complementation vector)	This study
pJIR3338	pJIR750 (SphI/BamHI) ΩJRP2244/JRP2825 PCR product (SphI/BamHI; 1,400 bp) ($tcpA_{1\text{-}316}$ complementation vector)	This study
pJIR3339	pJIR3096 $tcpA_{K242A}$	Site-directed mutagenesis
pJIR3340	pJIR750 (SphI/BamHI) ΩpJIR3339 (SphI/BamHI; 2,126 bp)	This study
pJIR3341	pJIR3096 $tcpA_{DE334/5AA}$	Site-directed mutagenesis
pJIR3342	pJIR750 (SphI/BamHI) ΩpJIR3341 (SphI/BamHI; 2,126 bp)	This study
pJIR3343	pJIR3096 $tcpA_{Q379A}$	Site-directed mutagenesis
pJIR3344	pJIR750 (SphI/BamHI) ΩpJIR3343 (SphI/BamHI; 2,126 bp)	This study
pJIR3345	pJIR3096 $tcpA_{\Delta 46aa\text{-}69}$	Site-directed mutagenesis
pJIR3346	pJIR750 (SphI/BamHI) ΩpJIR3345 (SphI/BamHI; 2,057 bp)	This study
pJIR3347	pJIR3096 $tcpA_{\Delta 79aa\text{-}104}$	Site-directed mutagenesis
pJIR3348	pJIR750 (SphI/BamHI) ΩpJIR3347 (SphI/BamHI; 2,051 bp)	This study
pJIR3349	pJIR3096 $tcpA_{\Delta 46aa\text{-}104}$	Site-directed mutagenesis
pJIR3350	pJIR750 (SphI/BamHI) ΩpJIR3349 (SphI/BamHI; 1,952 bp)	This study

[a] Rif^r, Nal^r, Tc^r, Em^r, Tm^R, Sm^r, and Chl^r, resistance to rifampin, nalidixic acid, tetracycline, erythromycin, thiamphenicol, streptomycin, and potassium chlorate, respectively.

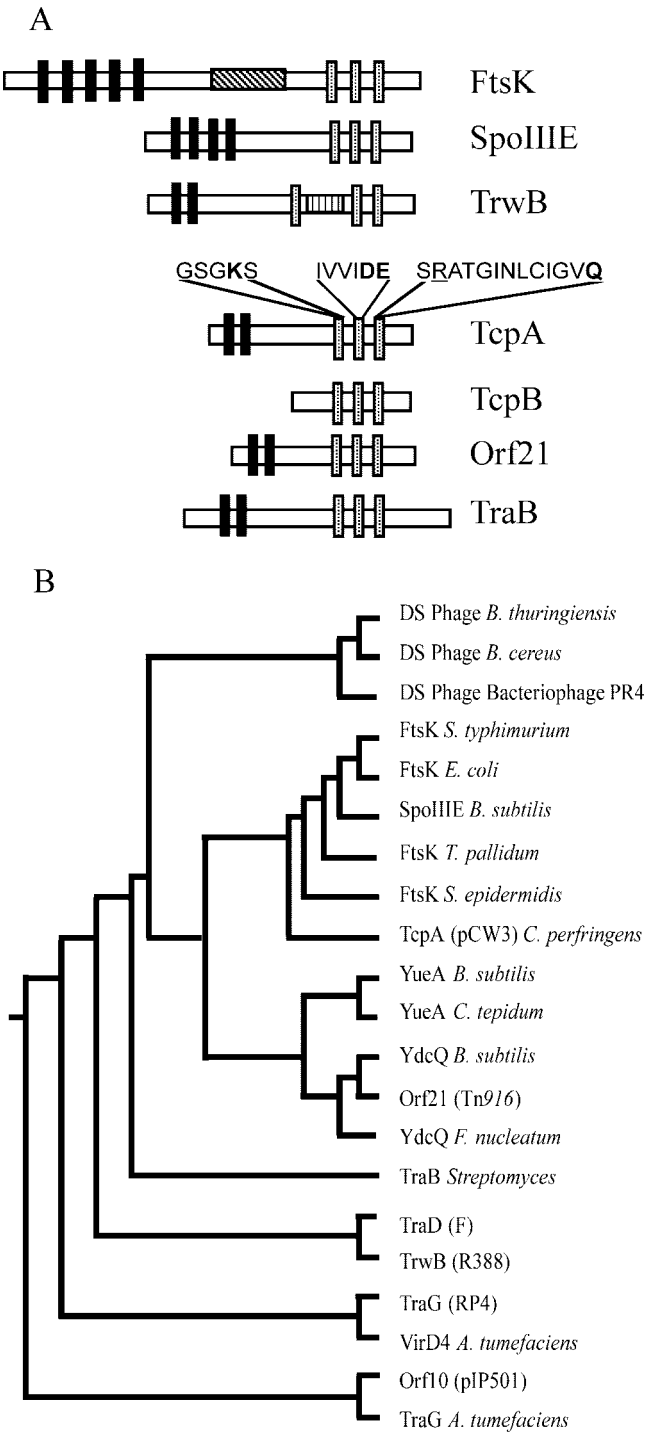

FIG. 1. Bioinformatic analysis of TcpA and TcpB. (A) Domain structure analysis of TcpA and TcpB. The analysis was performed using Sosui (24) and Conserved Domain Database (37) searches, and the results were compared with related FtsK-HerA ATPase proteins. TMDs are represented by solid bars; coiled-coil domains are represented by the box with diagonal stripes; the AAD region is represented by a box with vertical stripes; and the Walker A, Walker B, and RAAG motifs are represented by dotted bars. Residues of the TcpA motifs targeted by amino acid substitutions are in bold type, and the conserved arginine residue of the RAAG motif is underlined. (B) Dendrogram constructed using ClustalW alignments of TcpA, TcpB, and FtsK-HerA members and the PHYLIP algorithm (17).

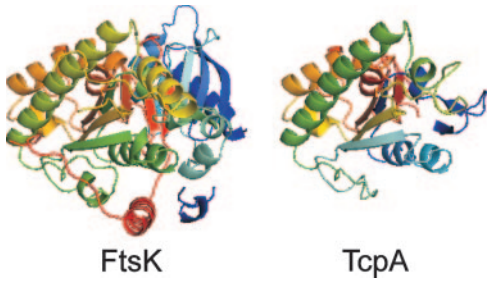

FIG. 2. Model of TcpA structure. Swiss Model (54) was used to construct a putative model of the central region of TcpA (aa 212 to 420). The template structure was a C-terminal region of FtsK (aa 818 to 1329; PDB: 2iusA) (38).

Multiple alignments of TcpA with various members of the FtsK-HerA superfamily suggested that TcpA was a member of this superfamily and was most similar to the FtsK clade. Members of this clade lack an AAD domain and typically have a coiled-coil region and a glycine before the core ATPase domain (26). The FtsK clade has been divided into three families, the classical FtsK family, the YueA family, and the YdcQ family. The distinction between the classical FtsK and YdcQ families is based on conserved residues, while YueA members contain three tandem ATPase domains. Searches of TcpA using these conserved features did not clearly identify TcpA as a member of any one of these families. To further define the relationships between the FtsK clade proteins and TcpA, ClustalW multiple alignments containing a diverse range of FtsK-HerA members were used. The resultant alignments indicated that TcpA branches with members of the FtsK clade (Fig. 1B). By using the crystal structure of an ATPase mutant of $FtsK_c$, a derivative of FtsK that contains the C-terminal region (38), a putative model (Fig. 2) of the central section of TcpA (aa 212 to 420) could be constructed using Swiss Model (54). This analysis supported the conclusion that TcpA is a member of the FtsK family. Note that TcpA could not be modeled on the known TrwB structure.

The *tcpB* gene, located directly downstream of *tcpA*, encodes a predicted 327-aa protein. Although conserved domain searches and BlastP analysis identified an FtsK domain within TcpB, the level of similarity was lower than that of TcpA. In addition, unlike other members of the FtsK family and the coupling proteins, TcpB lacks potential TMDs. Multiple alignments of TcpB with members of the FtsK-HerA superfamily indicated that the three conserved motifs were not well conserved in TcpB. The phylogenetic tree constructed using this alignment did not place TcpB within any of the described FtsK-HerA clades or families (data not shown), and TcpB could not be modeled on the FtsK structure.

TcpA is essential for the conjugative transfer of pCW3. Since *tcpA* and *tcpB* both encoded putative proteins with FtsK-like domains, pCW3Δ*tcpAB* deletion mutants were constructed by allelic exchange to investigate the role of these genes in the conjugative transfer of pCW3. After transformation of JIR325(pCW3) with the suicide vector pJIR3099, two independently derived deletion derivatives were obtained, and their pCW3Δ*tcpAB* genotypes were confirmed by PCR analysis and Southern blotting (data not shown). In mixed plate mat-

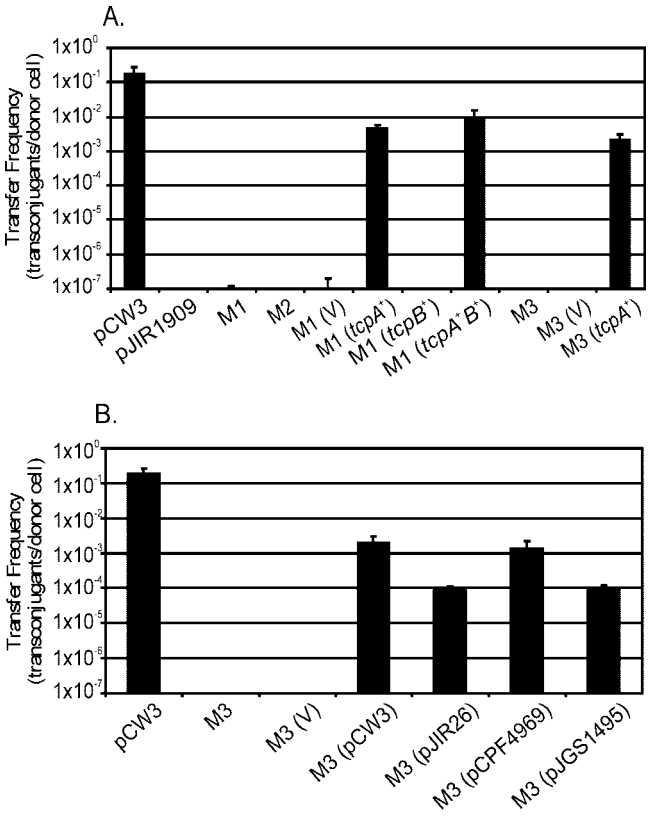

FIG. 3. Complementation of pCW3Δ*tcpAB* and pCW3Δ*tcpA* mutants. (A) Conjugation-deficient pCW3Δ*tcpAB* and pCW3Δ*tcpA* mutants were complemented with functional copies of *tcpAB*, *tcpA*, and *tcpB* and tested for the ability to transfer in mixed plate matings. (B) *tcpA* orthologs from pJIR26, pCPF4969, and pJGS1495 were tested for conjugative proficiency using mixed plate mating and compared to the wild-type pCW3 derivative. pCW3, wild-type positive control; pJIR1909, nontransferable negative control; M1, pCW3Δ*tcpAB1*; M2, pCW3Δ*tcpAB2*; M3, pCW3 Δ*tcpA*; V, pJIR750 vector control. The transfer efficiencies were expressed as the number of transconjugants per donor cell.

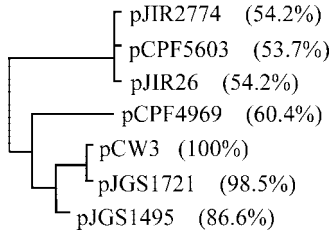

FIG. 4. Phylogenetic tree of TcpA homologs. The amino acid sequences of seven TcpA orthologs from a range of *C. perfringens* conjugative plasmids were aligned using ClustalW. The level of identity of each protein to TcpA from pCW3 is indicated in parentheses. The accession numbers are as follows: pCW3, DQ366035; pCPF4969, NC007772; pJIR2774, DQ338473; pCPF5603, NC007773; and pJIR26, DQ338471. The sequences of pJGS1495 and pJGS1721 were obtained from G. Myers, I. Paulsen, J. Songer, B. McClane, R. Titball, J. Rood, and S. Melville (personal communication).

ings these pCW3 derivatives (pJIR3101 and pJIR3102) were unable to confer conjugative transfer (Fig. 3). In these experiments the same host strain carrying pCW3 or pJIR1909 were used as positive and negative controls, respectively. Since both plasmids behaved in the same way, all further studies were carried out with pJIR3101.

To confirm that the loss of function was due to a specific mutation in pCW3, complementation analysis was performed. The wild-type *tcpA* and *tcpB* genes were cloned independently into the *C. perfringens-E. coli* shuttle vector pJIR750, as was a wild-type region containing both genes. The plasmids subsequently were introduced into JIR325(pJIR3101), which carried the mutated Δ*tcpAB*::*erm*(Q) region. Note that the replication region of pJIR750 is derived from the nonconjugative bacteriocin plasmid pIP404 and its derivatives are compatible with pCW3. Complementation with the wild-type *tcpAB* genes restored conjugative transfer, although not to wild-type levels (Fig. 3A). When the mutation was complemented with the wild-type *tcpA* gene, the conjugation frequency was not significantly different from that observed after complementation with the $tcpA^+B^+$ vector. By contrast, complementation with the wild-type *tcpB* gene alone did not restore conjugative transfer (Fig. 3A).

To confirm the essential role of the *tcpA* gene in conjugation, a pCW3Δ*tcpA* mutant was constructed by allelic exchange, shown to be nonconjugative, and then complemented with the $tcpA^+$ complementation vector. The transfer frequencies of pCW3Δ*tcpAB* and pCW3Δ*tcpA*, when complemented with the wild-type *tcpA* gene, were indistinguishable (Fig. 3A). Based on these results it was concluded that *tcpA* was essential for the conjugative transfer of pCW3 but that *tcpB* was not an essential conjugation gene.

Various *tcpA* homologs can complement the pCW3Δ*tcpA* mutation. The TcpA protein is the most variable gene product encoded in the *C. perfringens tcp* locus, and the levels of TcpA amino acid sequence identity vary from 54 to 98% (Fig. 4). In addition, several deletion and insertion events have been observed in the region between the *tcpA* and *tcpC* genes (8). The specificity of conjugation systems in gram-negative bacteria is determined by the interaction between the coupling proteins and the cognate relaxase (12). On this basis the abilities of several *tcpA* homologs to complement the pCW3Δ*tcpA* mutant were examined to investigate the functional relationship between the *tcp* loci of the most divergent conjugative plasmids from *C. perfringens*. A comparative analysis was carried out with TcpA homologs from the conjugative plasmids pCW3, pJIR2774 (which encodes lincomycin resistance), pJIR26 (which encodes tetracycline resistance), and pCPF4969 (which encodes the enterotoxin CPE), as well as plasmids pCPF5603 (CPE), pJGS1495 (β-toxin), and pJGS1721 (ε-toxin), which carry the *tcp* locus (8, 40) but whose conjugative abilities have not been tested. The resultant ClustalW alignments identified significant sequence identity in the N-terminal region of these proteins, as well as in the central region that encompasses the Walker boxes, although the last 70 aa were more variable (data not shown). The phylogenetic tree constructed from these alignments separated these TcpA homologs into four distinct groups (Fig. 4), which correlated with the genetic organization of the *tcp* region (8).

The genes encoding divergent TcpA homologs from pJIR26, pCPF4969, and pJGS1495 were cloned into pJIR750, and the resultant derivatives were introduced into *C. perfringens* and used to complement the pCW3Δ*tcpA* mutant in mixed plate

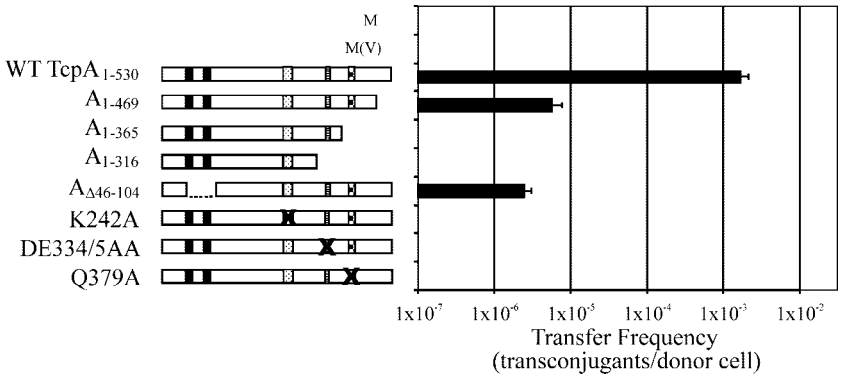

FIG. 5. Effect of deletion and site-directed mutations on TcpA activity. Isogenic shuttle vector derivatives were used to complement the pCW3Δ*tcpA* mutant (M), and the conjugation frequencies of the resultant derivatives were determined. The positive control carried the wild-type *tcpA* gene (WT $TcpA_{1-530}$), and the negative control carried the shuttle vector [M(V)]. The mutated *tcpA* derivatives encoded $TcpA_{1-469}$, $TcpA_{1-365}$, $TcpA_{1-316}$, $TcpA_{\Delta 46-69}$, $TcpA_{\Delta 79-104}$, $TcpA_{\Delta 46-104}$, TcpA $_{K242A}$, TcpA $_{DE334/5AA}$, and TcpA $_{Q379A}$. The solid bars represent TMDs, and the Walker A box, the Walker B box, and the RAAG motif are represented by dotted bars, bars with horizontal stripes, and checked bars, respectively. The crosses indicate the motifs targeted by site-directed mutagenesis.

matings. The *tcpA* gene from the CPE plasmid pCPF4969 yielded a transfer frequency that was indistinguishable from that obtained with the pCW3-derived *tcpA* gene. The genes from the tetracycline resistance plasmid pJIR26 and the β-toxin plasmid pJGS1495 also complemented the mutation, but at a frequency 1 order of magnitude lower (Fig. 3B). Comparative analysis could not attribute this reduction in transfer proficiency to any specific amino acid sequence variations.

Identification of the regions of TcpA that are essential for conjugation. To identify regions of TcpA that were required for the transfer of pCW3, a series of C-terminal deletion derivatives were constructed and tested for the ability to complement the pCW3Δ*tcpA* mutant (Fig. 5). We sequentially deleted the last 61 aa of TcpA ($TcpA_{1-469}$) and then the RAAG motif ($TcpA_{1-365}$) and the Walker B box ($TcpA_{1-316}$) specifically to investigate the role of these regions in the transfer of pCW3. Only one C-terminal deletion derivative, $tcpA_{1-469}$, restored transfer in the mutant, although not to levels observed with the full-length wild-type gene (Fig. 5). The other deletion derivatives, $tcpA_{1-365}$ and $tcpA_{1-316}$, were unable to complement the *tcpA* mutant. To investigate the role of the TMDs, derivatives of TcpA were constructed in which the putative TMDs were deleted individually and together. Both $tcpA_{\Delta 46-69}$ and $tcpA_{\Delta 76-104}$ were unable to complement the mutation (data not shown). However, a derivative with both TMDs deleted, $tcpA_{\Delta 46-104}$, was able to restore transfer, although not to wild-type levels (Fig. 5).

Since the TcpA Walker boxes were conserved, it was predicted that, like DNA translocases and coupling proteins, TcpA may have a requirement for ATPase activity. To investigate the role of the Walker boxes and the RAAG motif, individual site-directed mutants with substitutions in each motif were constructed and tested for the ability to complement the pCW3Δ*tcpA* mutant. No detectable transfer was observed for the derivatives containing alanine substitutions in the Walker A motif (K242A), the Walker B motif (DE334/335AA), or the RAAG motif (Q379A) (Fig. 5). Based on these results it was concluded that all three motifs are essential for the functional integrity of the TcpA protein.

DISCUSSION

Several *C. perfringens* toxin genes are associated with putative conjugative plasmids (8, 33, 40, 52), which has significant implications not only for the toxin-typing scheme used to type *C. perfringens* strains but also for pathogenesis. Despite this finding, very little is known about the effect of transfer on pathogenesis or the mechanism by which transfer occurs. To contribute to our understanding of the mechanism of *C. perfringens* conjugative transfer, we investigated the role of two genes in the conjugative transfer of the paradigm conjugative plasmid, pCW3. These genes, *tcpA* and *tcpB*, encode putative proteins with domains associated with DNA translocases, which suggests that either protein may be involved in the movement of DNA during conjugative transfer. In this study mutation and complementation analysis showed that pCW3Δ*tcpAB* mutants were unable to transfer by conjugation but could be complemented in *trans* with just the wild-type *tcpA* gene, providing evidence that loss of the ability to transfer was not the result of polarity effects. Furthermore, a pCW3Δ*tcpA* mutant was also transfer deficient and could be complemented with the *tcpA* gene. Based on these experiments we concluded that the *tcpA* gene has an essential role in the conjugative transfer of pCW3. *tcpA* is the third gene in the *tcp* locus shown to be essential for conjugation since previous studies have shown that both *tcpF* and *tcpH* are also essential conjugation genes (8).

Complementation of the pCW3Δ*tcpAB* mutants with the wild-type *tcpAB* genes had the same effect on conjugation as complementation with the *tcpA* gene alone. In addition, complementation with the *tcpB* gene alone did not restore conjugative transfer. These results provide good evidence that *tcpB* is not essential for transfer. The results of a comparative analysis of the *tcp* region from various conjugative plasmids from *C. perfringens* were in agreement with this conclusion since not all of these conjugative plasmids carry *tcpB*. Previous studies showed that *tcpB* is absent from the conjugative tetracycline resistance plasmid pJIR26, as well as from the β-toxin plasmid pJGS1495 (8). Furthermore, our analysis and that of other workers identified several potential recombination

events within the *tcpAB* region, including the presence of a third FtsK-like gene that is located between *tcpA* and *tcpB* in the conjugative CPE plasmid pCPF4969 and encodes a putative 203-aa protein (8, 40). It is postulated that *tcpB* is a truncated version of *tcpA* that arose from a gene duplication event and subsequent deletion events, although attempts to identify potential recombination sites by bioinformatic analysis have not been successful.

To investigate the relationship between TcpA, coupling proteins, and DNA translocases, multiple alignment was performed using members of the FtsK-HerA superfamily of ATPases. This family encompasses several families of proteins involved in the movement of DNA during bacterial replication, sporulation, conjugation, or phage packaging (26). Extensive analysis of this superfamily has identified conserved sequence blocks that encompass the ATPase domain and can be used to separate members of the superfamily into two superclades based on the presence of the AAD domain between the Walker A and Walker B boxes. Further classification of these superclades into clades and then families was carried out using residues located in and around the conserved sequence blocks. As determined by this approach, coupling proteins, which have an AAD domain, belong to a different superclade than the FtsK-SpoIIIE family of DNA translocases, which do not have an AAD domain (26). TcpA does not have the AAD region that is associated with coupling proteins and is most similar to members of the FtsK clade, to which part of TcpA can also be modeled. Members of this clade include proteins from gram-positive plasmids, bacteriophages, and conjugative transposons. These proteins are thought to function in *cis* as DNA pumps for the transfer of plasmid DNA during cell division or for packaging of DNA into the bacteriophage (26). ORF21 from the enterococcal conjugative transposon Tn*916* and the *Streptomyces* TraB protein are also FtsK-like proteins that lack the AAD region and are structurally similar to TcpA (Fig. 1). Although Tn*5* mutagenesis of ORF21 has implicated this protein in conjugative transfer (64), no data are available to shed light on its precise functional role.

In *Streptomyces*, TraB is a central factor in a conjugative transfer mechanism that is distinct from that of gram-negative bacteria. TraB is unique in that it is the only protein required for transfer (28). In this process, TraB binds specifically to *clt*, an essential 50-bp noncoding region adjacent to the *traB* gene (45). Analysis of denatured TraB-DNA complexes indicated that TraB does not process the DNA during binding (45), and furthermore, it is thought that dsDNA, not ssDNA, is transferred to the recipient cell (44). Since TcpA, like TraB, is also an FtsK-like protein and no pCW3-encoded relaxase has been identified, we cannot rule out the possibility that dsDNA rather than ssDNA is transferred during pCW3-mediated conjugation. However, pCW3 is unlikely to use a *Streptomyces*-like mechanism of transfer since the transfer of pCW3 also requires TcpH, a putative membrane-spanning protein, and TcpF, a putative cytoplasmic ATPase (8). It is postulated that although TcpA is more closely related to members of the FtsK family of dsDNA translocases, it is still a functional coupling protein that links the DNA to be transferred to the MPF apparatus.

Sequence analysis of plasmids that have the *tcp* locus has revealed that TcpA is the most variable *tcp* gene product (8). However, complementation with even the most divergent TcpA homologs was able to restore transfer proficiency to a pCW3Δ*tcpA* mutant (Fig. 3B). This result provides experimental evidence that supports the sequence data predictions that all of the conjugative plasmids that have been identified from *C. perfringens* use a similar transfer mechanism. Comparative analysis of these TcpA homologs identified significant C-terminal sequence variation, which may be attributable to recombination events that clearly occur in the *tcpAB* region. In the F plasmid system, changes to the C-terminal domain of TraD are associated with a decrease in the specificity of TraD, such that interactions occur with noncognate relaxases, although these changes also lead to a reduction in transfer efficiency (51). The *tcpA* genes from pJIR26 and pJGS1495 were less efficient at complementing a pCW3Δ*tcpA* mutant than the equivalent genes from pCW3 and pCPE4969. However, since the C-terminal region of TcpA from pJGS1495 is very similar to that from pCPF4969, sequence differences outside this region may also be responsible for the observed functional differences.

In other conjugation systems protein-protein interactions have been demonstrated between the relaxosome components TraM (F), TraI (RP4), and both TrwC and TrwA (R388) and their cognate coupling proteins (10, 35, 53). Interaction between TraM and the coupling protein TraD is dependent on the last 38 aa of TraD (10). To further investigate the role of the C terminus of TcpA in the transfer of pCW3, the deletion derivative $TcpA_{1\text{-}469}$ was constructed and analyzed. The results showed that removal of the last 61 aa led to a reduction in transfer frequency of at least 2 orders of magnitude (Fig. 4). Although this region of TcpA is not absolutely required for transfer, it clearly plays a major role in the conjugation process. Evidence for any involvement of this region with interactions between TcpA and the putative relaxase must await identification of the latter protein.

The Walker A box of the cytoplasmic domains of coupling proteins is essential for ATP binding and conjugative transfer (6, 30, 41). For example, in the *Agrobacterium tumefaciens* type IV secretion system the active transfer of the ssDNA-relaxase intermediate from VirB11 to the core subunits VirB6 and VirB8 is dependent on the Walker A box of VirD4 (4). Functional analysis of the Walker B motif in TraG (RP4) also has indicated that this motif is essential for conjugative transfer (6). The aspartate residue targeted for substitution in TraG (and in TcpA in this study) is predicted to be required for coordination of the magnesium cation (Mg^{2+}) involved in nucleoside triphosphate hydrolysis, whereas the neighboring glutamate primes a water molecule for a nucleophilic attack on the γ-phosphate of the bound ATP (60). To date no functional analysis of the RAAG motif of coupling proteins has been reported; however, comparative analysis of the structural similarity between TrwB and F_1-ATPase has indicated that the conserved arginine of this motif may function as an arginine finger (21). In AAA^+ ATPases such as F_1-ATPase the arginine finger is thought to interact with the C-terminal phosphate of an ATP molecule that is bound to the preceding protomer in the hexameric ring and to affect oligomerization, ATP recognition, and ATP hydrolysis (14, 43). The conserved glutamine appears to be required for sensing the triphosphate moiety of the bound nucleotide, triggering its hydrolysis (29, 60). In this study, substitutions in each of these three motifs in TcpA eliminated conjugative transfer, providing evidence that TcpA-

mediated ATPase activity is essential for the conjugation process.

The TMDs of TraG are essential for transfer of RP4; they are required for oligomerization in vitro, as well as protein-protein interactions with relaxosome components (53). By contrast, the TMDs of FtsK are not essential for its ATPase activity, multimer formation, or DNA translocase activity (5). Similarly, we have found that the TMDs of TcpA are not essential for transfer, although deletion of these TMDs led to a very significant reduction in transfer frequency (Fig. 5). Protein-protein interactions between the TMD deletion derivative $TcpA_{\Delta 46\text{-}104}$ and other conjugation proteins may still allow correct localization of TcpA at the MPF apparatus, although at a greatly reduced efficiency.

In this study we demonstrated that TcpA is essential for the conjugative transfer of pCW3. Functional analysis of TcpA confirmed the role of the Walker A and B boxes, the RAAG motif, and the TMDs and suggested that efficient transfer depends upon the ATPase activity of TcpA and its membrane localization. Given the relationship between TcpA and the FtsK-HerA family of pumping ATPases, we propose that TcpA acts as a functional coupling protein and therefore is involved in the movement of DNA during conjugative transfer. By carrying out similar studies with the other Tcp proteins and analyzing their abilities to interact with TcpA, we aim to develop a model that describes the conjugation process in *C. perfringens*. Since all of the known conjugative plasmids from *C. perfringens* have the *tcp* locus and therefore appear to transfer by the same Tcp-mediated mechanism and since the *tcp* locus is also present on several other toxin plasmids not yet demonstrated to transfer (8, 33, 40, 52), the development of this model is critical for our understanding of how toxin genes and antibiotic resistance determinants can move between strains of this important human and animal pathogen.

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