Two Novel Membrane Proteins, TcpD and TcpE, Are Essential for Conjugative Transfer of pCW3 in Clostridium perfringens

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The anaerobic pathogen Clostridium perfringens encodes either toxin genes or antibiotic resistance determinants on a unique family of conjugative plasmids that have a novel conjugation region, the tcp locus. Studies of the paradigm conjugative plasmid from C. perfringens, the 47-kb tetracycline resistance plasmid pCW3, have identified several tcp-encoded proteins that are involved in conjugative transfer and form part of the transfer apparatus. In this study, the role of the conserved hypothetical proteins TcpD, TcpE, and TcpJ was examined. Mutation and complementation analyses showed that TcpD and TcpE were essential for the conjugative transfer of pCW3, whereas TcpJ was not required. To analyze the TcpD and TcpE proteins in C. perfringens, functional hemagglutinin (HA)-tagged derivatives were constructed. Western blots showed that TcpD and TcpE localized to the cell envelope fraction independently of the presence of other pCW3-encoded proteins. Finally, examination of the subcellular localization of TcpD and TcpE by immunofluorescence showed that these proteins were concentrated at both poles of C. perfringens donor cells, where they are postulated to form essential components of the multiprotein complex that comprises the transfer apparatus.

Conjugative transfer of plasmid DNA is an important process that contributes to bacterial genome plasticity and adaption, particularly the spread of antibiotic resistance and virulence genes (1, 2). The translocation of a conjugative plasmid relies on the coupling protein, the relaxosome complex, and a type IV secretion system (T4SS), known as the mating-pair formation (Mpf) apparatus (1). For conjugation systems in Gram-negative bacteria, structural resolution of the outer membrane core complex and the inner membrane complex of the T4SS, as well as information about the pathway of DNA translocation across the T4SS in the donor cell, has been elucidated (3–11). Less is known about conjugation systems in Gram-positive bacteria, with our current understanding based primarily on comparisons to those in Gram-negative bacteria and with the systems in Gram-positive bacteria proposed to involve a “minimized” T4SS (3, 12–18).

The Gram-positive, anaerobic pathogen Clostridium perfringens carries many disease-mediating toxin genes and antibiotic resistance genes on a unique family of large plasmids that have large regions of sequence identity (19–22). Most of these plasmids carry the novel tcp conjugation locus (20, 23). The tcp locus has been identified on all known conjugative plasmids in C. perfringens, and proteins encoded by this locus have been shown to be required for conjugative transfer of the paradigm C. perfringens conjugative plasmid, pCW3 (20, 23–26). The presence of the tcp genes on so many of these toxin plasmids suggests that the transfer of toxin genes by a shared conjugation mechanism in C. perfringens is important in disease dissemination. To this effect, several of these toxin plasmids have been shown experimentally to be conjugative (19, 27, 28).

Mutagenesis and biochemical studies of the Tcp proteins encoded on pCW3 have led to the development of a model of the C. perfringens transfer apparatus (26, 29). Surprising similarities between the components that form the C. perfringens transfer apparatus and the T4SS in Gram-negative bacteria have been identified (26, 29). The C. perfringens transfer apparatus is composed of the core components TcpH and TcpC, which contain domains from VirB6- and VirB8-like proteins that are part of the inner membrane complex of the T4SS in Gram-negative bacteria (26, 30). Furthermore, protein families that are found in conjugation systems in Gram-negative and Gram-positive bacteria are involved in conjugative transfer in C. perfringens (13). These components include two putative hexameric ATPases that are essential for transfer, the coupling protein TcpA and a transmembrane protein, TcpF, as well as a peptidoglycan hydrolase, TcpG, which is required for optimal transfer efficiency (23–25).

The objective of this study was to determine whether hypothetical proteins encoded by the tcp locus were required for the conjugative transfer of pCW3. Three genes encoding the hypothetical proteins TcpD, TcpE, and TcpJ, are highly conserved in tcp loci from C. perfringens plasmids (20, 23). The role of these conserved proteins in conjugative transfer remains unknown, since they have no similarity to proteins of known function (23). In the current study, conjugative mating studies carried out on tcpD, tcpE, and tcpJ mutants of pCW3, and their complemented derivatives, demonstrated that TcpD and TcpE were essential for conjugative transfer, whereas TcpJ was not required. Furthermore, TcpD and TcpE were shown to be membrane-associated proteins that concentrated at the poles of C. perfringens cells, similar to previous findings for TcpH and TcpF (30). These findings support the hy-
TABLE 1  C. perfringens strains and plasmids

<table>
<thead>
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<th>Strain or plasmid</th>
<th>Characteristic(s)</th>
<th>Reference(s) or source</th>
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<tr>
<td>JIR325</td>
<td>Strain 13 derivative; Rif r NaI r</td>
<td>58</td>
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<tr>
<td>JIR4195</td>
<td>JIR325(pCW3); Rif r NaI r Tc r</td>
<td>28</td>
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<tr>
<td>JIR394</td>
<td>Strain 13 derivative; Sm r Chl r</td>
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<td>JIR12540</td>
<td>JIR325(pJIR3760); Rif r NaI r Tc r Em r</td>
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Plasmids

- pCW3  Conjugative tetracycline resistance plasmid
- pJIR2715  Base plasmid for construction of C. perfringens suicide vectors; erm(Q)-catP T oriT
- pJIR3405  JIR2715(Xhol/SacI) JRP3664/JRP3665 (Xhol/SacI; 2,002 bp, pCW3) (downstream of tcpD)
- pJIR3406  JIR3405(Asp718/BamHI) JRP3663/JRP3662 (Asp718/BamHI; 1,867 bp, pCW3)
- pJIR3407  JIR3407(BamHI/Asp718) JRP3660/JRP3661 (BamHI/Asp718; 432 bp, pCW3)
- pJIR3408  JIR3407(Asp718/BamHI) JRP3659/JRP3658 (Asp718/BamHI; 1,999 bp, pCW3)
- pJIR3414  JIR3414(XhoI/SacI) JRP3705/JRP3706 (XhoI/SacI; 1,906 bp, pCW3) (upstream of tcpP)
- pJIR3415  JIR3415(Xhol/SacI) JRP3707/JRP3708 (Xhol/SacI; 1,992 bp, pCW3) (tcpP suicide vector)
- pJIR3422  E. coli-C. perfringens shuttle vector; catP r lacZ r
- pJIR3440  pCW3ΔtcpD::erm(Q)
- pJIR3442  pCW3ΔtcpE::erm(Q)
- pJIR3444  pCW3Δ(tcpD::erm(Q))
- pJIR3614  pJIR3422(BamHI/Asp718) JRP4049 (BamHI/Asp718; 424 bp, pCW3)
- pJIR3757  pJIR3422(BamHI/Asp718) JRP4489 (BamHI/Asp718; 451 bp, pCW3)
- pJIR3759  pJIR3422(BamHI/Asp718) JRP4484 (BamHI/Asp718; 432 bp, pCW3)
- pJIR4203  pJIR3422(BamHI/Asp718) JRP4046 (BamHI/Asp718; 405 bp, pCW3)
- pJIR4204  pJIR3422(BamHI/Asp718) JRP4728 (BamHI/Asp718; 450 bp, pCW3)

- JIR12212: JIR325(pJIR3440); Rif r NaI r Tc r Em r
- JIR12215: JIR325(pJIR3443); Rif r NaI r Tc r Em r
- JIR12216: JIR325(pJIR3444); Rif r NaI r Tc r Em r
- JIR12540: JIR325(pJIR3760); Rif r NaI r Tc r Em r

- Reference(s) or source
- J. A. Wisniewski, T. L. Bannam, and J. I. Rood, unpublished data

**Note**: Rif r, rifampin resistance; NaI r, nalidixic acid resistance; Tc r, tetracycline resistance; Em r, erythromycin resistance; Sm r, streptomycin resistance; Chl r, potassium chlorate resistance.

**MATERIALS AND METHODS**

**Bacterial strains, plasmids, and culture conditions.** Bacterial strains and plasmids are described in Table 1. C. perfringens strains were cultured at 37°C in preboiled fluid thioglycolate (FTG) medium (Difco), TPGY broth (31), brain heart infusion broth or agar (Oxoid), or nutrient agar (32) supplemented with glucose (0.38%, wt/vol) (Amersco) and the appropriate antibiotics following sterilization. The following antibiotics were used (at the final concentrations indicated in parentheses): erythromycin (50 μg/ml), streptomycin (1 mg/ml), tetracycline (10 μg/ml), and thiamphenicol (10 μg/ml). Chlorate-resistant strains were selected on medium containing a 1% (vol/vol) saturated potassium chlorate solution. C. perfringens agar cultures were incubated in an atmosphere of 10% (vol/vol) H₂, 10% (vol/vol) CO₂, and 80% (vol/vol) N₂ in an anaerobic jar (Oxoid). *Escherichia coli* host strains DH15α and DH125 (Life Technologies) were cultured at 37°C in 2YT medium (33) supplemented with chloramphenicol (30 μg/ml), erythromycin (150 μg/ml), or kanamycin (20 μg/ml) where appropriate.

**Molecular techniques.** Plasmid DNA from E. coli was isolated by using an alkaline lysis method according to the manufacturer’s instructions (Qiagen). E. coli cells were made chemically competent and transformed as previously described (34). Electroporant cells were prepared for E. coli (35) and C. perfringens (36) as previously described, with slight modifications. TPGY medium was used to culture *C. perfringens*, and cells were...
washed three times with SMP buffer (270 mM sucrose, 1 mM MgCl₂, 7 mM Na₂HPO₄, [pH 7.3]). Transformation of electrocompetent cells was carried out by using a BTX ECM-630 Electro Cell Manipulator (BTX Laboratories) with a single pulse of 1.8 kV, a resistance of 200 Ω, and a capacitance of 25 μF. C. perfringens genomic DNA was extracted as previously described (37). PCR amplification was performed by using Taq or Pwo DNA polymerase (Roche) and a final concentration of 0.5 μM each primer. PCR products were purified by using a QIAquick PCR purification kit (Qiagen). Restriction enzymes were used according to the manufacturer’s instructions (Roche and New England BioLabs). Sequencing was performed by using Prism BigDye Terminator mix (Applied Biosystems) and was carried out on an Applied Biosystems 3730S capillary sequencer. Sequences were analyzed by using Vector NTI (Invitrogen) and Sequencher 5.1 (Gene Codes Corporation). Amino acid sequences were analyzed and putative functional domains were identified by using PSI-BLAST (38), TopPred2 (39), PSORT Prediction (40), and TOPCONS (41).

Construction of mutants by allelic exchange and complementation.

Allelic exchange was performed by using a C. perfringens suicide plasmid, pJIR2715, containing an erm(Q) erythromycin resistance cassette that was flanked by ca. 2 kb of sequence upstream and downstream of the gene to be mutated (23). The flanking regions were generated by PCR and sequentially cloned into pJIR2715 using Asp718/BamHI sites upstream and XhoI/Sacl sites downstream of the (erm)Q gene. The pCW3 region from bp 27700 to 29567 (23) was cloned upstream of (erm)Q, and the region from bp 29895 to 31897 was cloned downstream of (erm)Q, to generate the tcpD suicide vector pJIR3406. The pCW3 regions from bp 27941 to 29940 and from 30244 to 32234 were cloned upstream and downstream, respectively, of (erm)Q to generate the tcpE suicide vector pJIR3408. Similarly, the pCW3 regions from bp 35904 to 37000 and from bp 37719 to 39711 were cloned upstream and downstream, respectively, of (erm)Q to generate the tcpJ suicide vector pJIR3415. The suicide vectors were introduced independently into JIR325(CW3) cells by electroporation. DNA preparations from potential double-crossover events were identified as erythromycin-resistant, thiamphenicol-susceptible colonies and were tested by PCR and Southern blotting to confirm the replacement of the target gene(s) with the (erm)Q cassette and the loss of the suicide plasmid.

For complementation studies, PCR products carrying either the wild-type tcpD or tcpE gene and ca. 50 bp of upstream sequence were cloned into the BamHI/Asp718 sites of the E. coli-C. perfringens shuttle vector pJIR3422 (24, 24) to generate pJIR4203 and pJIR3614, respectively. To generate the C-terminal hemagglutinin (HA)-tagged tcpE complementation vector, the wild-type tcpE gene and ca. 50 bp of upstream sequence were PCR amplified by using a reverse primer that encoded the HA sequence. The PCR product was cloned into the BamHI/Asp718 sites of pJIR3422 to generate pJIR3757. To generate the C-terminal HA-tagged tcpD complementation vector, the wild-type tcpD gene and ca. 50 bp of upstream sequence were PCR amplified by using a reverse primer that encoded a hexaglycine polylinker and the HA sequence. The PCR product was cloned into the BamHI/Asp718 sites of pJIR3422 to generate pJIR4204. The complementation plasmids were confirmed by restriction endonuclease and sequence analyses. These plasmids were introduced into C. perfringens by electroporation, and transformants were selected on nutrient agar containing thiamphenicol. The presence of the complementation plasmids in the C. perfringens strains was confirmed by restriction endonuclease and sequence analyses after rescuing the plasmids in E. coli.

Conjugative transfer experiments.

Mixed plate matings on solid medium were performed to test conjugative plasmid transfer from C. perfringens donor cells, as previously described (32, 43). The recipient used in all mating studies was a streptomycin- and potassium chlorate-resistant derivative of strain 13, JIR4394 (23). The conjugation frequency is reported as the number of transconjugants per donor cell. To perform statistical analysis, the conjugation frequencies were log transformed, and an unpaired t test was performed on the normalized data, with statistical significance being defined as a P value of <0.05.

Cell fractionation studies.

Crude membrane preparations of C. perfringens cells were prepared essentially as described previously (26, 30). Cells resuspended in phosphate-buffered saline (PBS) (10 mM sodium phosphate, 1.8 mM potassium phosphate, 137 mM NaCl, 2.7 mM KCl [pH 7.4]) supplemented with Complete cocktail protease inhibitor (Roche) were disrupted by using an Emulsiflex-C5 high-pressure homogenizer (Avestin). The protein content of the cellular fractions was determined by using a bicinchoninic acid (BCA) assay (Pierce) in accordance with the manufacturer’s instructions. Approximately 2 μg of each fraction was analyzed by performing SDS-PAGE followed by Coomassie staining and Western blotting using anti-HA mouse monoclonal antibodies (Cell Signaling) and horseradish peroxidase-conjugated goat anti-mouse antibodies (Millipore).

Immunofluorescence studies.

Immunofluorescence analysis of HA-tagged Tcp proteins was performed according to a previously reported method, with modifications (44). A single colony of each C. perfringens strain was used to inoculate 6 ml of FTG broth, with selection, and grown overnight at 37°C. The resultant cultures were subcultured into 90 ml of TPGY broth with appropriate antibiotics and incubated for 4 to 5 h. Cells (500 μl) were fixed in 10 ml of methanol (Amersco) for 1 to 2 h at −20°C and then centrifuged at 4,000 × g for 5 min at 4°C. Cells were then washed with PBS and resuspended in 90 μl of GTE (50 mM glucose, 20 mM Tris-HCl [pH 7.5], and 10 mM EDTA). A solution containing lysozyme (Amersco) and lysozymin (Sigma) was freshly prepared in GTE and added to the cell suspension at final concentrations of 1 mg/ml and 10 μg/ml, respectively. The cell suspension was incubated for 2 h at 37°C, and 10 μl was then spread onto a 0.1% poly-I-lysine (Sigma)-treated slide. The cells were incubated with anti-HA mouse monoclonal antibodies (1:100 dilution in 2% [wt/vol] bovine serum albumin [Sigma] in PBS) overnight at 4°C. After washing 10 times with PBS, the cells were treated with 5% (vol/vol) goat serum (Sigma) in PBS for 30 min at room temperature. The cells were then incubated in the dark with 2 μg/ml Alexa Fluor 488–goat anti-mouse secondary antibody (Molecular Probes) in the presence of 300 nM 4′,6′-diamidino-2-phenylindole (DAPI; Molecular Probes) in PBS at room temperature for 1 to 2 h. The cells were then washed 10 times with PBS and covered with a coverslip using 50% glycerol (Amersco) in PBS. The stained cells were observed by using an Olympus BX60 microscope with a 100× oil immersion objective (Olympus). Images were captured by using a SPOT RT3 digital camera with SPOT Basic for image capture (Scitech, Australia). Images were pseudocolored (green for Alexa Fluor 488 and blue for DAPI) and merged for analysis and preparation for publication by using Image J (45).

RESULTS

TcpD and TcpE are essential for pCW3 conjugative transfer.

To determine whether the hypothetical proteins encoded by tcpD, tcpE, and tcpJ were required for conjugative transfer, null deletion mutants of pCW3 were constructed by allelic exchange for each of these genes independently. The mutants were selected on the basis of the erythromycin resistance conferred by the (erm)Q gene (Fig. 1A), with the genotypes of the mutants being confirmed by PCR analysis and Southern blotting (data not shown). The ability of the pCW3Δtcp derivatives to confer conjugative transfer was determined by a mixed plate mating assay that detected the transfer of the tetracycline resistance encoded by pCW3 to an isogenic tetracycline-sensitive recipient strain. The pCW3Δtcp/l mutant transferred at levels similar to those of the wild type (Fig. 1B), suggesting that tcpJ is not required for conjugative transfer. In contrast, no transfer was detected for either the pCW3ΔtcpD or pCW3ΔtcpE mutant (Fig. 1B).

To confirm that the transfer-deficient phenotype observed for the tcpD and tcpE mutants was due to the allelic replacement of the
transmembrane domains (TMDs) as the only known functional domains. Three putative TMDs were identified in TcpD, spanning the majority of the protein sequence (residues 20 to 40, 54 to 74, and 78 to 98), while two N-terminal putative TMDs were identified in TcpE (residues 31 to 51 and 59 to 79). The identification of putative TMDs suggests that TcpD and TcpE may localize to the cell membrane in *C. perfringens* cells, in a manner similar to that of other Tcp proteins that form the *C. perfringens* transfer apparatus (26, 30).

To determine the location of TcpD and TcpE in *C. perfringens* cells, the wild-type tcpD and tcpE genes were extended at their 3’ ends with a small, 9-amino-acid hemagglutinin (HA) epitope-encoding tag by PCR. These tagged tcpD and tcpE genes were introduced into the respective *C. perfringens* pCW3ΔtcpD and pCW3ΔtcpE mutants on shuttle plasmid pJIR3422. To determine whether the TcpD-HA and TcpE-HA derivatives were functional, mixed plate matings were carried out with the mutants complemented with the HA-tagged derivatives. Complementation with tcpE-HA restored transfer in the pCW3ΔtcpE mutant to levels similar to those found for complementation with the wild-type tcpE gene (Fig. 1B), providing evidence that the HA-tagged TcpE protein was functional. In contrast, complementation with the tcpD-HA derivative did not restore transfer in the pCW3ΔtcpD mutant (data not shown). This result indicated that the TcpD-HA protein was nonfunctional and therefore cannot be used to study TcpD localization. To produce a functional TcpD-tagged derivative, a hexaglycine (G6) flexible linker was introduced between the C terminus of TcpD and the HA tag. Complementation with the tcpE-HA derivative restored transfer in the pCW3ΔtcpD mutant to levels similar to those found for complementation with wild-type tcpD (Fig. 1B). Based on these results, we concluded that a functional tagged TcpD derivative had been constructed.

To determine the cellular location of TcpD and TcpE, cellular fractionation was performed on cell lysates from *C. perfringens* pCW3ΔtcpD or pCW3ΔtcpE mutant strains expressing the wild-type TcpD and TcpE proteins from the complementation plasmids and the equivalent strains carrying the TcpD-G6-HA or TcpE-HA complementation plasmids. In addition, we examined an isogenic pCW3-negative *C. perfringens* strain expressing TcpD-G6-HA or TcpE-HA. Analysis of whole-cell lysates by SDS–PAGE and immunoblotting with HA-specific monoclonal antibodies detected bands at the expected masses of 12.7 kDa and 14.5 kDa for TcpD-G6-HA and TcpE-HA, respectively (Fig. 2). No bands were observed for lysates expressing the wild-type TcpD and TcpE proteins (data not shown). Bands corresponding to the TcpD-G6-HA and TcpE-HA proteins were observed in the cell lysate and insoluble cell membrane fractions in the presence and absence of pCW3, with no bands being observed in the soluble fraction (Fig. 2). These results provide evidence that TcpD and TcpE are membrane-associated proteins that localize to the cell membrane independently of other pCW3-encoded proteins.

The levels of TcpD-G6-HA detected in the cell lysate and insoluble cell membrane fraction of pCW3-negative *C. perfringens* cells were consistently lower than the those detected in pCW3-positive *C. perfringens* cells (Fig. 2). To determine whether this reduction was the result of a mutation in the shuttle plasmid, the TcpD-G6-HA plasmid was rescued from the pCW3-negative *C. perfringens* strain into *E. coli* and then introduced into the isogenic pCW3-positive *C. perfringens* pCW3ΔtcpD strain. The levels of TcpD-G6-HA detected in the strain carrying the rescued plasmid

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**FIG 1** Conjugation frequencies of pCW3 mutants and the respective complemented derivatives. (A) Region of the tcp locus spanning tcpC to tcpE from the wild type (pCW3) and the pCW3ΔtcpD::erm(Q) and pCW3ΔtcpE::erm(Q) mutants. In the mutants, most of the tcpD or tcpE gene has been replaced with the erm(Q) gene. (B) Conjugation frequencies of the pCW3ΔtcpD, pCW3ΔtcpE, and pCW3Δtcp mutants and their respective complementation derivatives. Donors shown on the x axis are isogenic strains in a JIR325 background. pCW3, wild-type pCW3 (JIR4195); ΔtcpD, pCW3ΔtcpD (JIR12216); ΔtcpE, pCW3ΔtcpE (JIR12212); ΔtcpDΔ tcpE (V), JIR12212 with a vector plasmid (pJIR3422); ΔtcpD tcpE (Δ’), JIR12212 with a vector plasmid carrying the wild-type tcpD gene (pJIR4203); ΔtcpD tcpE (Δ’’), JIR12212 with a vector plasmid carrying the tcpD HA-tagged derivative (pJIR4204); ΔtcpE, pCW3ΔtcpE (V), JIR122125 with a vector plasmid (pJIR3422); ΔtcpE tcpE (Δ’’’), JIR1221215 with a vector plasmid carrying the wild-type tcpE gene (pJIR3614); ΔtcpE tcpE HA (Δ’’’’), JIR1221215 with a vector plasmid carrying the tcpE HA-tagged derivative (pJIR3577). The transfer frequency is expressed as the number of transconjugants per donor cell. The means ± standard errors of the means are shown, based on results from at least three biological replicates. Statistical analysis was carried out by using an unpaired t test. One asterisk denotes statistical significance (P < 0.05) compared to wild-type pCW3 (positive control). Two asterisks denote statistical significance (P < 0.05) when complemented strains were compared to the respective vector control (V) strain.

respective genes, the wild-type genes were introduced into the respective mutant derivatives in *trans* under the control of a clostridial promoter on shuttle plasmid pJIR3422. Conjugative transfer of the pCW3 derivatives was restored in both complemented strains (Fig. 1B), leading to the conclusion that both tcpD and tcpE are essential for conjugative transfer of pCW3.

**TcpD and TcpE localize to the cell envelope fraction of *C. perfringens* donor cells.** TcpD and TcpE are small proteins of 115 and 122 amino acids, respectively, with TcpE having 27% sequence identity to the ORF17 protein of unknown function from *Tn916*, the only similarity identified (23). Bioinformatic analysis of the amino acid sequences of TcpD and TcpE identified putative

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were similar to those previously observed for pCW3-positive *C. perfringens* cells expressing TcpD-G6-HA from the shuttle plasmid (Fig. 2). We conclude that the reduced TcpD levels observed in the absence of pCW3 are not due to a forced mutation in the shuttle plasmid. These results could be explained by postulating that TcpD-G6-HA is less stable in the absence of other pCW3-encoded proteins.

To determine whether the stability of TcpD-G6-HA requires the presence of one of the other Tcp proteins, the TcpD-G6-HA shuttle plasmid was introduced into each of our *C. perfringens* tcp mutant backgrounds; that is, the shuttle plasmid was used to transform separate *intP* (J. A. Wisniewski and J. I. Rood, unpublished data) tcpA (25), tcpC (26), tcpE (this study), tcpF (23), tcpG (24), and tcpH (23) mutants. Western blotting of fractionated cell membrane preparations of these strains showed that the levels of TcpD-G6-HA detected in the various tcp mutant backgrounds were similar to those observed in the pCW3ΔtcpD mutant (Fig. 3). We conclude that no single Tcp protein is required to stabilize TcpD in *C. perfringens* cells.

**TcpD and TcpE are concentrated at the poles of *C. perfringens* cells.** Previous studies have shown that Tcp proteins that are presumed to be part of the transfer apparatus, TcpH and TcpF, are localized to the poles of *C. perfringens* cells (30). To determine the subcellular localization of TcpD and TcpE, immunofluorescence microscopy was used to detect the HA-tagged derivatives in *C. perfringens* cells, indicating that the fluorescence observed was due to the presence of the HA epitope (Fig. 4). The polar localization of TcpD and TcpE suggests that these proteins are components of the *C. perfringens* transfer apparatus located or concentrated at the poles of donor cells. However, bacterial two-hybrid analysis did not detect interactions between TcpD and TcpE and the other Tcp proteins (29, 30; J. A. Wisniewski, R. Bantwal, T. L. Bannam, and J. I. Rood, unpublished data).

**DISCUSSION**

The tcp region is essential for conjugative transfer of pCW3 and is present on all known conjugative toxin and resistance plasmids from *C. perfringens*, suggesting that these plasmids utilize a similar conjugation mechanism (20). Determination of the functional role and protein-protein interactions of several Tcp proteins has resulted in the development of a model of the *C. perfringens* transfer apparatus (20). A recent classification of T4SSs involved in conjugation identified the conjugation region from pCW3 as belonging to the Mpf_Fa class that includes Tn916 and other integrative and conjugative elements from the *Firmicutes* and *Actinobacteria* (46). Several specific protein profiles were identified in this family, including the unique TcpE protein family, which lacked homologs to components from other conjugation T4SS classes.

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**FIG 2** Localization of TcpD and TcpE HA-tagged derivatives in *C. perfringens* cells. Cell fractionation and Western blotting using HA antibodies were carried out on strains expressing either the TcpD or TcpE HA-tagged derivative in a pCW3-positive (+) or pCW3-negative (−) background. pTcpD-HA denotes the vector plasmid carrying the tcpD HA-tagged derivative (pJIR4204), and pTcpD-G6-HA(R) denotes the vector plasmid carrying the tcpD HA-tagged derivative (pJIR4204) rescued from a pCW3-negative background. Expression was examined in a JIR325(pCW3)-positive background in the pCW3ΔtcpE (JIR12215) mutant or the pCW3ΔtcpD (JIR12212) mutant or in a pCW3-negative background in JIR325. The fractions are labeled as follows: L, cell lysate; S, soluble fraction; I, insoluble cell membrane fraction.

**FIG 3** Localization of TcpD HA-tagged derivatives in *C. perfringens* tcp mutant backgrounds. Cell fractionation and Western blotting using HA antibodies were carried out on isogenic derivatives of *C. perfringens* strain JIR325 expressing the TcpD HA-tagged derivative (pTcpD-G6-HA) in a pCW3-positive or pCW3-negative background as well as in tcp mutant backgrounds. pTcpD-G6-HA denotes the vector plasmid carrying the tcpD HA-tagged derivative (pJIR4204) expressed in JIR325 (−) and isogenic strains carrying pCW3 derivatives, abbreviated as follows: ΔD, pCW3ΔtcpD (JIR12212); intP::TT, pCW3intP::TT (JIR12540); ΔA, pCW3ΔtcpA (JIR10251); ΔC, pCW3ΔtcpC (JIR12088); ΔE, pCW3ΔtcpE (JIR12215); ΔF, pCW3ΔtcpF (JIR4940); ΔG, pCW3ΔtcpG (JIR12128); ΔH, pCW3ΔtcpH (JIR4685). The fractions are labeled as follows: L, cell lysate; S, soluble fraction; I, insoluble cell membrane fraction.
TcpD and TcpE Are C. perfringens Conjugation Proteins

To further define the mechanism of conjugation in C. perfringens, and other conjugation systems from the MPF \(_\text{FA}\) class, we examined three highly conserved hypothetical proteins that are encoded by all known tcp regions. TcpD, TcpE, and TcpJ are small proteins of unknown function, with the putative TMDs in TcpD and TcpE being the only structural or functional domains identified. Mutagenesis performed in this study showed that both the pCW3\(\Delta tcpD\) and pCW3\(\Delta tcpE\) mutants were unable to transfer. Conjugative transfer was restored by complementation with the respective wild-type genes in the absence of pCW3-encoded proteins, confirming that the tcpD gene is not required for TcpD stability, we suggest that the key difference may be that for TcpD expression from the vector, suggesting that the TcpD protein was less stable in the absence of the other pCW3-encoded proteins. Similarly, others have shown that mutations in VirB4, VirB7, or VirB8 result in a loss of VirB3 accumulation, while VirB9 fails to accumulate to wild-type levels in the absence of VirB7 (51, 53). Recent findings have also shown that the accumulation of the TrwK (VirB4) and TrwD (VirB11) ATPases from the R388 T4SS was dependent on the presence of other core components of the R388 T4SS (34). Since analyses of TcpD levels in tcp mutant backgrounds did not identify any specific Tcp protein that was necessary for TcpD stability, we suggest that the key difference may be the absence of either a Tcp protein complex or an unknown pCW3-encoded factor.

We previously showed that essential conjugation proteins predicted to be key components of the transfer apparatus, TcpH and TcpF, colocalize at the poles of C. perfringens donor cells (30). Based on these findings, it was postulated that the C. perfringens transfer apparatus is formed at the poles, similar to the T4SS from A. tumefaciens (30, 55–57). We now provide evidence that the TcpD and TcpE proteins also are concentrated at the poles of C. perfringens cells. Coupled with our finding that, like TcpH and TcpF (30), TcpD and TcpE are cell membrane associated, we postulate that these proteins form an essential component of the C. perfringens transfer apparatus in the cell membrane.

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