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Characterization and Localization of the *Campylobacter jejuni* Transformation System Proteins CtsE, CtsP, and CtsX

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The human pathogen *Campylobacter jejuni* is naturally competent for transformation with its own DNA. Genes required for efficient transformation in *C. jejuni* include those similar to components of type II secretion systems found in many Gram-negative bacteria (R. S. Wiesner, D. R. Hendrixson, and V. J. DiRita, J Bacteriol 185:5408–5418, 2003, http://dx.doi.org/10.1128/JB.185.18.5408-5418.2003). Two of these, *ctsE* and *ctsP*, encode proteins annotated as putative nucleotide binding nucleoside triphosphatases (NTPases) or nucleoside triphosphate (NTP) binding proteins. Here we demonstrate that the nucleotide binding motifs of both proteins are essential for their function in transformation of *C. jejuni*. Localization experiments demonstrated that CtsE is a soluble protein while CtsP is membrane associated in *C. jejuni*. A bacterial two-hybrid screen identified an interaction between CtsP and CtsX, an integral membrane protein also required for transformation. Topological analysis of CtsX by the use of LacZ and PhoA fusions demonstrated it to be a bitopic, integral membrane protein with a cytoplasmic amino terminus and a periplasmic carboxyl terminus. Notwithstanding its interaction with membrane-localized CtsX, CtsP inherently associates with the membrane, requiring neither CtsX nor several other Cts proteins for this association.

The Gram-negative bacterium *Campylobacter jejuni* is a leading cause of bacterial gastroenteritis worldwide (1). *C. jejuni* often colonizes the avian intestinal tract; consequently, a common route of infection is through consumption of contaminated poultry (2).

A number of *Campylobacter* species are naturally competent for transformation, meaning that they can take up macromolecular DNA from the environment and incorporate it heritably into their genomes (3, 4). The ability to acquire DNA from the environment may contribute to the extensive genetic diversity observed among strains of *C. jejuni* (5, 6). Horizontal gene transfer *in vivo* has been demonstrated during experimental infection of chicks, a natural host for this pathogen (7).

Multiple genes whose products are involved in natural transformation of *C. jejuni* have been identified (8–12). Using transposon mutagenesis and a genetic screen for loss of competence, we isolated mutations that mapped to 11 genes encoded in *C. jejuni* strain 81-176 (8). Mutations in these result in a reduction in transformability to levels 4 orders of magnitude below the levels seen with the wild type (8). Among these are six genes arranged in a likely operon, some of which encode proteins similar to components of type II secretion and type IV pilus biogenesis systems and homologous to proteins important for natural transformation in other organisms (13). Two of these, *ctsE* and *ctsP*, encode putative nucleoside triphosphatases (NTPases) or nucleoside triphosphate (NTP) binding proteins, according to the annotated genome of *C. jejuni* strain 11168 (8, 14).

CtsE is a member of the type II secretion/type IV secretion system superfamily of NTPases collectively referred to as the PulE-VirB11 family (15, 16). Members of this family are involved in diverse processes, including secretion, pilus biogenesis, competence for natural transformation, and conjugation (15). PulE-VirB11 family members share several elements, including the nucleotide-binding motifs—Walker boxes A and B—an Asp box, and a His box (17–19). CtsE also has a tetracysteine motif conserved among the GspE and PilB/HofB subfamilies (20, 21).

ATPase activity has been demonstrated *in vitro* for several members of the PulE-VirB11 family (22–26). It is hypothesized that this activity powers the transformation process, though this has yet to be conclusively shown. Phylogenetic analyses placed CtsE in the type II secretion subfamily of NTPases; in one such analysis, CtsE fell between the ComG1 subfamily of Gram-positive NTPases involved in competence and the PilT and PilU subfamily involved in retraction of type IV pili (15, 16). Another analysis placed CtsE in the Gram-positive competence subfamily, closely related to the GspE subfamily of type II secretion machinery (16). Although ATPase activity has not been demonstrated for most of the type II secretion machinery, it has been hypothesized.

CtsE is one of two putative identified NTP binding proteins important for natural transformation of *C. jejuni*. The other is CtsP (8), which has Walker box A and B nucleotide binding motifs but lacks other characteristics of the PulE-VirB11 superfamily, including the His box, the Asp box, and the tetracysteine motif. According to BLAST analyses, CtsP exhibits weak homology to several ATPases, including PilT homologues, ClpX ATP binding subunits, and members of the AAA family of ATPases. The Walker

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TABLE 1 Strains and plasmids used in this study

Strain or plasmid	Relevant characteristic(s)[a]	Source or reference
Strains		
E. coli DH5α	*supE44* Δ*lacU169* (ϕ80*lacZ*ΔM15) *hsdR17 recA1 endA1 gyrA96 thi-1 relA1*	Laboratory strain
E. coli JM101	F′ *traD36 proA*$^+$*B*$^+$ *lacI*^{q}*Z*ΔM15 Δ(*lax-proAB*) *glnV thi*	New England Biolabs
E. coli DH5α pRK212.1	Contains conjugative plasmid for transferring DNA into *Campylobacter*	28
E. coli Sp850	λ$^-$ *e14*$^-$ *relA1 spoT1 cyaA1400*(::*kan*) *thi-1*	*E. coli* Genetic Stock Center, Yale
E. coli TG1	Δ*hsdS* Δ(*lac-proAB*) *supE thi* [F^0*traD36 proAB*$^+$ *lacI*^{q}*Z*ΔM15]	Laboratory strain
C. jejuni 81-176	Clinical isolate	29
C. jejuni DRH153	81-176 *astA*::*aphA3*; Kn^r	30
C. jejuni DRH212	81-176 *rpsL*^{Sm}	30
C. jejuni RSW46	DRH212 *ctsX*	8
C. jejuni RSW53	81-176 *ceuB*::*solo*	8
C. jejuni RSW57	81-176 *ctsD*::*solo*	8
C. jejuni RSW58	81-176 *ctsF*::*solo*	8
C. jejuni RSW115	DRH212 *ctsP*	8
C. jejuni RSW136	DRH212 *ctsE*	8
Plasmids		
pT18	Encodes T18 fragment of CyaA	31
pT25	Encodes T25 fragment of CyaA	31
pT18-Zip	Encodes T18 fragment in frame with 35-aa leucine zipper	31
pT25-Zip	Encodes T25 fragment in frame with 35-aa leucine zipper	31
pTrcLacZ	pTrc99A with the *lacZ* gene (lacks codons Met 1 to Ser 7)	32
pTrcPhoA	pTrc99A with the *phoA* gene (lacks codons Met 1 to Met 26)	32
pECO102	pRY112 derivative with *cat* promoter in XhoI-BamHI sites	8
pRSW176	pT18 with *ctsP* in XhoI-ClaI sites	This study
pRSW184	pT25 with *ctsX* in PstI-BamHI sites	This study
pRSW186	pT25 with *ctsE* in PstI-BamHI sites	This study
pRSW191	pT18 with *ctsE* in XhoI-ClaI sites	This study
pRSW206	pECO102 with N-terminal His tag in frame with BamHI site	This study
pRSW208	pECO102 with *ctsP* and a C-terminal FLAG tag	
pRSW211	pECO102 with C-terminal FLAG tag in frame with XhoI site	This study
pRSW223	pRSW211 with *ctsE* in BamHI-XhoI site	This study
pRSW228	pRSW208 with E81A point mutation	This study
pRSW246	pRSW223 with K296Q point mutation	This study
pRSW248	pTrcLacZ with coding sequence for 1st 50 aa of *ctsX* in NcoI-XmaI site	This study
pRSW249	pTrcLacZ with coding sequence for 1st 100 aa of *ctsX* in NcoI-XmaI site	This study
pRSW250	pTrcPhoA with coding sequence for 1st 14 aa of *ctsX* in NcoI-XmaI site	This study
pRSW251	pTrcLacZ with coding sequence for 1st 14 aa of *ctsX* in NcoI-XmaI site	This study
pRSW252	pTrcPhoA with coding sequence for 1st 50 aa of *ctsX* in NcoI-XmaI site	This study
pRSW253	pTrcPhoA with coding sequence for 1st 100 aa of *ctsX* in NcoI-XmaI site	This study
pRSW254	pTrcPhoA with coding sequence of *ctsX* in NcoI-XmaI site	This study
pRSW255	pTrcLacZ with coding sequence of *ctsX* in NcoI-XmaI site	This study
pRSW256	pRSW211 with *ctsX* in BamHI-XhoI sites	This study
pJMB1	pRSW206 with *ctsX* in BamHI-XhoI	This study

[a] aa, amino acids; Kn, kanamycin.

boxes of CtsP resemble those of the AAA$^+$ superfamily, but CtsP does not appear to have the minimal AAA consensus sequence (18, 27).

In this study, we characterized CtsE and CtsP, the two putative NTPases/NTP binding proteins required for natural transformation of *C. jejuni*. We also carried out analysis of a third gene product encoded in the *cts* gene cluster, CtsX, unique to the *C. jejuni* transformation system. CtsX lacks significant sequence homology to other proteins and shares no clearly conserved domains beyond a transmembrane domain. We determined the subcellular localization of each of these proteins, and we investigated the roles of the nucleotide binding motifs in CtsE and CtsP. Further analysis investigated protein-protein interactions among constituents of the type II-like Cts system in *C. jejuni* (8), revealing association between CtsP and CtsX.

MATERIALS AND METHODS

Bacterial strains and media. Bacterial strains used in this work are listed in Table 1. *C. jejuni* was routinely cultured on Mueller-Hinton (MH) agar with 10% sheep's blood under microaerophilic conditions (5% CO_2, 10% O_2, balanced with N_2) in a trigas incubator at 37°C. When necessary, media were supplemented with the following antibiotics at the indicated concentrations: trimethoprim (10 μg ml^{-1}), kanamycin (as noted; either 30 or 150 μg ml^{-1}), streptomycin (100 μg ml^{-1}), and chloramphenicol

TABLE 2 Oligonucleotide primers used in this study

Plasmid	Sequence[a]	Site	Primer name[b]
pRSW176	5′ CCC*ATCGAT*ACCCTTAATAAGCCATTCTCTAAAGCACTAAGCTTT 3′	ClaI	Cj1473c pT18 3′
pRSW176	5′ CCG*CTCGAG*GAGTAAAATTATTCCATTTAGAGAAGAAATTTTTC 3′	XhoI	Cj1473c pT18 5′
pRSW184	5′ CGC*GGATCC*CTACTTTCCATCTAATTCCATTAAACC 3′	BamHI	Cj1472c pT25 3′
pRSW184	5′ AAAA*CTGCAG*GGCAAGAAAGAATTAAAGAGCTTGAGCTTAGG *3′*	PstI	Cj1472c pT25 5′
pRSW186	5′ CGC*GGATCC*TCATCTTACAACCCTTAAAAGCTCATC 3′	BamHI	Cj1471c pT25 3′
pRSW186	5′ AAAA*CTGCAG*GGGAAAGTAGAATGGATAAAATTTTTCAAGC 3′	PstI	Cj1471c pT25 5′
pRSW191	5′ CCC*ATCGAT*ACTCTTACAACCCTTAAAAGCTCATCTATAC 3′	ClaI	Cj1471c pT18 3′
pRSW191	5′ CCG*CTCGAG*GGAAAGTAGAATGGATAAAATTTTTCAAGC 3′	XhoI	Cj1471c pT18 5′
pRSW208	5′ CGC*GGATCC*AGTAAAATTATTCCATTTAGAGAAGAA 3′	BamHI	Cj1473c comp strt
pRSW208	5′ CCG*CTCGAG*TTATTTGTCATCATCGTCCTTATAATCCCTTAATAAGCCATTCTC TAAAGCACT 3′	XhoI	FLAG
pRSW211	5′ ATCAAGCTTATCGATACCGTCGACCTCGAGGATTATAAGGACGATGATGA CAAATAAGGGGGGCCCGGTACCCAGCTTTT3′		pECO102 FLAG tag 1
pRSW211	5′ AAAAGCTGGGTACCGGGCCCCCCTTATTTGTCATCATCGTCCTTATAATCCT CGAGGTCGACGGTATCGATAAGCTTGAT3′		pECO102 FLAG tag 2
pRSW223	5′ CGC*GGATCC*CAAAGTAGAATGGATAAAATTTTTCAAGCT 3′	BamHI	Cj1471c comp strt
pRSW223	5′ CCG*CTCGAG*TCTTACAACCCTTAAAAGCTCATCTAT 3′	XhoI	Cj1471c no stp cod
pRSW228	5′ CAAGGTCAGAGTTAAATCATTCTTGATGCAGTGGGAATGTATGATTATGCAA3′		CtsP E81A 1
pRSW228	5′ TTGCATAATCATACATTCCCACTGCATCAAGAATGATTAAACTCTGACCTTG3′		CtsP E81A 2
pRSW246	5′ AAACACGCGTATAAAGTTGTGCTTTGACCACTACCTGTAGGACCGGTTAAT3′		Cj1471c K296Q 1
pRSW246	5′ATTAACCGGTCCTACAGGTAGTGGTCAAAGCACAACTTTA TACGCGTGTTT3′		Cj1471c K296Q 2
pRSW248	5′ CTCATC*CATGG*CGCAAGAAAGAATTAAAGAGCTTGAGCTT 3′	NcoI	CtsX fusion 5′
pRSW248	5′ TCCC*CCCGGG*AATATTTTTTTGCTTATTATATTTTTGCAT 3′	XmaI	CtsX 50 aa fusion 3′
pRSW249	5′ CTCATC*CATGG*CGCAAGAAAGAATTAAAGAGCTTGAGCTT 3′	NcoI	CtsX fusion 5′
pRSW249	5′ TCCC*CCCGGG*TTTAACTTCTTCTAGTTCTTTATATAACTT 3′	XmaI	CtsX 100 aa fusion 3′
pRSW250	5′ ACAATTTCACACAGGAAACAGACCATGCAAGAAAGAATTAAAGAGCTTGAGCT TAGGTATAAATACTTTCCTGTTCTGGAAAAC CGGGCTGCTCAG 3′		Nterm 14aa PhoA1
pRSW250	5′ CTGAGCAGCCCGGTTTTCCAGAACAGGAAAGTATTTATACCTAAGCTCAAGCT CTTTAATTCTTTCTTGCATGGTCTGTTTCCTGTG TGAAATTGT 3′		Nterm 14aa PhoA2
pRSW251	5′ ACAATTTCACACAGGAAACAGACCATGCAAGAAAGAATTAAAGAGCTTGAGCT TAGGTATAAATACTTTCTGGCCGTCGTTTTACA ACGGGGATCC 3′		Nterm 14aa LacZ1
pRSW251	5′ GGATCCCCGTTGTAAAACGACGGCCAGAAAGTATTTATACCTAAGCTCAAGCT CTTTAATTCTTTCTTGCATGGTCTGTTTCCT GTGTGAAATTGT 3′		Nterm 14aa LacZ2
pRSW252	5′ CTCATC*CATGG*CGCAAGAAAGAATTAAAGAGCTTGAGCTT 3′	NcoI	CtsX fusion 5′
pRSW252	5′ TCCC*CCCGGG*AATATTTTTTTGCTTATTATATTTTTGCAT 3′	XmaI	CtsX 50 aa fusion 3′
pRSW253	5′ CTCATC*CATGG*CGCAAGAAAGAATTAAAGAGCTTGAGCTT 3′	NcoI	CtsX fusion 5′
pRSW253	5′ TCCC*CCCGGG*TTTAACTTCTTCTAGTTCTTTATATAACTT 3′	XmaI	CtsX 100 aa fusion 3′
pRSW254	5′ CTCATC*CATGG*CGCAAGAAAGAATTAAAGAGCTTGAGCTT 3′	NcoI	CtsX fusion 5′
pRSW254	5′ TCCC*CCCGGG*CTTTCCATCTAATTCCATTAAACCATA 3′	XmaI	CtsX full fusion 3′
pRSW255	5′ CTCATC*CATGG*CGCAAGAAAGAATTAAAGAGCTTGAGCTT 3′	NcoI	CtsX fusion 5′
pRSW255	5′ TCCC*CCCGGG*CTTTCCATCTAATTCCATTAAACCATA 3′	XmaI	CtsX full fusion 3′
pRSW256	5′ CGC*GGATCC*CAAGAAAGAATTAAAGAGCTTGAGCTT 3′	BamHI	Cj1472c comp strt
pRSW256	5′ CCG*CTCGAG*CTTTCCATCTAATTCCATTAAACCATA 3′	XhoI	Cj1472c no stp cod
pJMB1	5′ CGC*GGATCC*AGTAAAATTATTCCCATTTAGAGAAGAA 3′	BamHI	CtsX comp strt + BamHI
pJMB1	5′ CCG*CTCGAG*TTACCTTAATAAGCCATTCTCTAAAGA 3′	XhoI	CtsX comp no stp cod +XhoI

[a] Italic sequence characters represent restriction enzyme cut sites.
[b] Abbreviations: aa, amino acids; comp strt, complement start codon; no stp cod, no stop codon.

(as noted; either 20 or 30 μg ml^{-1}). All *C. jejuni* strains were stored at −80°C in MH broth with 20% glycerol.

Escherichia coli strains were routinely cultured at 37°C in Luria-Bertani (LB) broth or agar. When necessary, the following antibiotics were used at the indicated concentrations: ampicillin (100 μg ml^{-1}), chloramphenicol (30 μg ml^{-1}), kanamycin (50 μg ml^{-1}), and tetracycline (12.5 μg ml^{-1}). All *E. coli* strains were stored at −80°C in LB broth–20% glycerol.

Construction of FLAG fusion proteins. To express FLAG fusion proteins in *C. jejuni*, we modified pECO102 by mutagenesis with PFU turbo (8). Two primers were made that annealed to pECO102 and would insert the coding sequence for the FLAG tag and a stop codon downstream of the XhoI site. (All primers used in this study are indicated in Table 2). pECO102 was amplified by PCR with these primers, and the template DNA was digested with DpnI. *E. coli* DH5α was transformed with the reaction mixture, and clones were screened by PCR. Candidate plasmids were sequenced to confirm insertion of the FLAG sequence, and one, pRSW211, was used for subsequent cloning. To construct in-frame fusion proteins with a C-terminal FLAG tag, PCR primers were designed to amplify the coding sequence of the protein of interest from the second amino acid through the last amino acid (excluding the stop codon). The 5′ primer had BamHI sites and the 3′ primer had XhoI sites for in-frame insertion into pRSW211. All subsequent clones were verified by sequence determination.

Construction of Walker box point mutants. Point mutants were constructed in the Walker boxes of *ctsE* and *ctsP* by PFU mutagenesis (Stratagene). Both *ctsE* and *ctsP* were cloned into pRSW211 to create C-terminal FLAG fusions pRSW223 (CtsE-FLAG) and pRSW208 (CtsP-FLAG). The

conserved lysine residue (K296) of Walker box A of CtsE-FLAG was mutated by changing the coding sequence from AAA to CAA, resulting in an amino acid change to a glutamine (K296Q); this plasmid was designated pRSW246. The conserved glutamic acid residue (E81A) of Walker box B of CtsP-FLAG was mutated by changing the coding sequence from GAA to GCA, resulting in an amino acid change to an alanine (E81A); this plasmid was designated pRSW228. DH5α pRK212.1 was transformed with these constructs, and conjugations were performed as described previously (28, 33). pRSW208 and pRSW228 were conjugated into strain DRH212 Δ*ctsP* (RSW115), while pRSW223 and pRSW246 were conjugated into strain DRH212 Δ*ctsE* (RSW136) (8). Transconjugants were verified by PCR. Expression of the fusion proteins was detected in whole-cell lysates by SDS-PAGE and Western blot analysis as discussed below.

Construction of His fusion proteins. To express a 6×His-tagged version of CtsX, genomic DNA from *C. jejuni* strain 81-176 was used as a template to amplify *ctsX* from the second codon to the stop codon using a forward primer with a BamHI site and a reverse primer with an XhoI site flanking the coding sequence. This fragment was cloned into the BamHI and XhoI sites of pBW206, a derivative of pECO102 that contains an N-terminal 6×His tag immediately upstream of the BamHI site, creating JMB1. This plasmid was transformed into DH5α pRK212.1 and transferred by conjugation into the Δ*ctsX* strain as described previously (28, 33). Transconjugants were verified by PCR, and the stability of the fusion proteins was assessed using SDS-PAGE and Western blot analysis of whole-cell lysates.

Transformation assays. Transformation assays were performed as described previously (3, 8). Briefly, *C. jejuni* was grown 16 to 18 h on MH agar plates supplemented with appropriate antibiotics. Cells were resuspended in MH broth to an optical density at 600 nm of 0.5, and 500-μl aliquots were added to 13-mm-long test tubes containing 1 ml of solidified agar. After incubation for 3 h at 37°C in 5% CO_2, 1 μg of DRH153 (81-176 *astA*::*aphA3*) DNA was added and the cultures were incubated for an additional 4 h (30). The number of transformants and the total number of bacteria were determined by dilution plating on MH agar with appropriate antibiotics. Transformation efficiency values represent the number of transformants per total number of bacteria per microgram of DNA. Transformations were conducted in triplicate, and the transformation efficiency data represent averages of the results from three samples from one experiment.

Cell fractionations. Cell fractionation was carried out as previously described for *C. jejuni* with a few modifications (34). *C. jejuni* was cultured on MH agar plates under microaerophilic conditions at 37°C for 16 to 18 h. Cells were resuspended from plates in MH broth and centrifuged (10,000 × *g*, 10 min at 4°C), and the resulting pellet was resuspended in 10 mM HEPES (pH 7.4). After one freeze-thaw cycle in a dry ice-ethanol bath, cells were sonicated with a microtip in a Branson digital sonifier at 30% amplitude six times for 10 s each time. Cell debris was pelleted by centrifugation (10,000 × *g* for 10 min at 4°C in a Sorvall tabletop centrifuge), and the supernatants were subjected to ultracentrifugation to form membrane pellets (100,000 × *g* for 70 min at 4°C with an SW41 rotor). The supernatant was removed and saved as the soluble fraction; the pellet (membrane fraction) was washed once in 10 mM HEPES and subsequently resuspended in an appropriate volume of 10 mM Tris-HCl (pH 8.0). The soluble fraction contains cytoplasmic and periplasmic contents, while the membrane fraction contains both inner and outer membranes. The same procedure was followed for fractionation of *E. coli*. Protein concentrations were determined with the Bio-Rad protein assay or with the ThermoScientific BCA assay per the manufacturer's instructions. Equal concentrations of proteins were then analyzed by SDS-PAGE and Western blot analysis.

Membrane flotation. *C. jejuni* was cultured on MH agar plates under microaerophilic conditions at 37°C for 16 to 18 h. Cells were resuspended from plates in MH broth and centrifuged (Sorvall tabletop centrifuge; 10,000 × *g* for 10 min at 4°C), and the resulting pellet was resuspended in P buffer (100 mM sodium phosphate [pH 7.6], 50 mM $MgCl_2$, 10 mM EDTA). Cells were lysed by passage through a French press. Lysates were incubated with DNase for 30 min and centrifuged (5,000 rpm for 5 min at 4°C) to remove cell debris. The supernatant was subjected to ultracentrifugation (100,000 × *g* for 1 h at 4°C; SW41 rotor), and a sample of the supernatant, containing the cytosolic and periplasmic proteins, was collected. The pellet was resuspended in P buffer and centrifuged (100,000 × *g* for 1 h at 4°C; SW41 rotor). The supernatant was discarded, and the pellet was resuspended in P buffer and mixed with 81% sucrose (dissolved in P buffer) to reach a final concentration of 71%. The sample was overlaid with 52% and 42% sucrose and subjected to ultracentrifugation (18 h at 100,000 × *g* and 4°C; SW41 rotor). After ultracentrifugation, the presence of the membrane bands was noted. Fractions (1.8 ml) were taken, starting from the top of the sample, and were labeled 1 to 6 sequentially. The sucrose was diluted in P buffer, and proteins were precipitated using 2% deoxycholate and trichloroacetic acid (TCA) overnight at 4°C. After the proteins were precipitated, fractions were subjected to ultracentrifugation (1.5 h at 100,000 × *g* and 4°C; SW41 rotor). Pellets from each fraction were washed with acetone to remove residual TCA and subsequently with 100% ether to remove lipids. Pellets were vacuum dried and resuspended in water for testing in the isocitrate dehydrogenase assay or resuspended in 6× sample buffer for SDS-PAGE and immunoblot analysis.

SDS-PAGE and immunoblotting. To detect FLAG fusion proteins, samples were diluted 1:1 with 2× SDS sample buffer and boiled for 5 min before being loaded onto 12% polyacrylamide gels for separation by SDS-PAGE. For His-CtsX-containing samples, samples were diluted 1:6 with 6× SDS sample buffer and were separated on a 15% polyacrylamide gel. Samples were normalized by protein concentration. Proteins were transferred to nitrocellulose membranes (Hoefer semidry; 200 mA for 2 h), and, after blocking with 5% milk–Tris-buffered saline (TBS), membranes were probed with primary antibody. For FLAG-tagged fusion proteins, an anti-FLAG M2 monoclonal antibody–peroxidase conjugate (Sigma) was used at 1:1,000. This was developed using Western Lighting Chemiluminescence Reagent Plus (Perkin-Elmer Life Sciences). For the His-tagged fusion protein, THE His tag antibody (Genscript) was used at 1:1,500. A rat anti-mouse IgG1 horseradish peroxidase (HRP)-conjugated antibody (Southern Biotech) was used at 1:3,500 for the secondary antibody, and blots were developed using SuperSignal West Pico chemiluminescent substrate (ThermoScientific).

Isocitrate dehydrogenase assays. To demonstrate efficient fractionation, fractions were tested for the presence of isocitrate dehydrogenase, a cytoplasmic protein. Isocitrate dehydrogenase activity was assayed as described previously (34). The reaction mixture contained 100 μl 10 mM $MgCl_2$, 100 μl 50 mM Tris-HCl (pH 8.0), 100 μl 10 mM NADP, 550 to 600 μl distilled H_2O, and 0 to 50 μl of the cell fraction. The reaction was started by the addition of 100 μl of 50 mM sodium isocitrate, and the optical density at 340 nm was read at 15-s intervals for 3 min. The specific activity of isocitrate dehydrogenase was calculated using an absorption coefficient of NADPH of 6.22 mM^{-1} cm^{-1} at 340 nm. The percentage (of the total unfractionated sample) of isocitrate dehydrogenase activity present in each fraction was calculated. For all fractionations, a minimum of 90% of the isocitrate dehydrogenase activity was present in the cytoplasmic fraction.

Identification of interacting proteins with the bacterial-two hybrid system. A bacterial two-hybrid system was used to identify potential interactions among Cts proteins. This system is based on functional complementation between two fragments of the catalytic domain of adenylate cyclase from *Bordetella pertussis* (31). Genes *ctsE*, *ctsP*, *ctsX*, *ctsF*, *ctsD*, *ctsR*, *ctsG*, *ctsW*, and *dprA* were PCR amplified from the coding sequence of the second amino acid through the stop codon and cloned into the PstI and BamHI sites of pT25 to generate in-frame T25 fusion proteins. The same genes were amplified with primers containing XhoI and ClaI sites from the second amino acid to the last amino acid, excluding the stop codon, and inserted into the XhoI and ClaI sites of pT18 to create in-frame fusion proteins corresponding to the adenylate cyclase T18

domain. The insertions in these plasmids were verified by PCR and confirmed by sequencing.

E. coli strain Sp850 (*cya*) was transformed with all combinations of Cts-pT18 and Cts-pT25 plasmids, and transformants were screened for protein-protein interactions by growth on LB agar plates at 30°C with 100 μg ml^{-1} ampicillin, 30 μg ml^{-1} chloramphenicol, 40 μg ml^{-1} X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside), and 0.5 mM ITPG (isopropyl-β-D-thiogalactopyranoside). The negative control contained Sp850 with pT18 and pT25, and the positive control was Sp850 with pT18-Zip and pT25-Zip (31). Assays were done in triplicate, and the data represent averages of the results of three biological replicate experiments. Standard deviations are indicated.

Topology analysis of CtsX. LacZ and PhoA fusions of *ctsX* were made using pTrcLacZ and pTrcPhoA (32). Translational fusions were generated at amino acid residues 14, 50, and 89 and at amino acid residue 195 (the final amino acid in the full-length CtsX protein). These sites were chosen after analysis of CtsX hydrophobicity by the use of several computer programs (Kyte Doolittle, EMBL) which predicted a transmembrane segment at roughly amino acids 22 to 42. PCR products were cloned into the NcoI and XmaI sites in pTrcLacZ and pTrcPhoA. Plasmids were screened by PCR and verified by sequencing. *E. coli* strain TG1 was transformed with these plasmids to carry out alkaline phosphatase assays and β-galactosidase assays using standard methods (35). Strains were assayed in triplicate, and data represent averages of the results of three biological replicate experiments. Standard deviations are indicated.

Membrane extractability of CtsP and CtsX fusion proteins. For membrane solubility studies, cells were fractionated as described previously. Purified membranes were diluted 1:1 into (i) 10 mM Tris-HCl (pH 8.0), (ii) 1 M NaCl, (iii) 2 M NaCl, (iv) 3 M urea, (v) 5 M urea, (vi) 0.5 M Na_2CO_3 (pH 3.0), (vii) 0.5 M Na_2CO_3 (pH 11.0), or (viii) 1 M KCl and rocked on a Nutator mixer for 30 min at 4°C. Membranes were subsequently collected by ultracentrifugation (1 h at 100,000 × *g* and 4°C; SW41 rotor) and were resuspended in 50 μl of 10 mM Tris (pH 8.0). The supernatant was removed and precipitated by addition of 400 μl of acetone, incubation at −20°C for 1 h, and centrifugation at 16,000 × *g* at 4°C. Precipitated proteins were air dried for 30 min and resuspended in 50 μl of 10 mM Tris-HCl (pH 8.0). Equal volumes of soluble and membrane fractions were resuspended in SDS sample buffer and loaded onto polyacrylamide gels for separation by SDS-PAGE and Western blot analysis to detect CtsX or CtsP as described above.

RESULTS

The Walker boxes of CtsE and CtsP are required for function *in vivo*. Walker boxes A and B are conserved nucleotide binding motifs important for NTPase function (19), and both motifs are present in CtsP and CtsE. To determine the contribution of CtsP and CtsE for natural transformation of *C. jejuni*, we tested whether Walker box motifs are important *in vivo*. Carboxyl-terminal FLAG fusions to each protein were expressed in *C. jejuni* Δ*ctsP* and Δ*ctsE* mutants; the fusion proteins complemented the respective mutant alleles to near-wild-type levels of transformation, similar to the complementation observed with untagged alleles of *ctsP* or *ctsE* (Fig. 1) (8).

Walker box A (GXXXXGK[S/T], where X is any amino acid) forms a loop structure (the P-loop) in which the lysine can directly contact the phosphoryl group of the bound nucleotide (36, 37). This Walker box motif is important for the function of many PulE-VirB11 superfamily members, and mutation of invariant residues abolishes ATP binding in a number of proteins (17, 38–40). The conserved lysine codon (K296) of Walker box A in *ctsE* was changed by site-directed mutagenesis to encode a glutamine in CtsE-FLAG (CtsE-FLAG K296Q), which was expressed in a Δ*ctsE* strain to determine whether it could restore transformation.

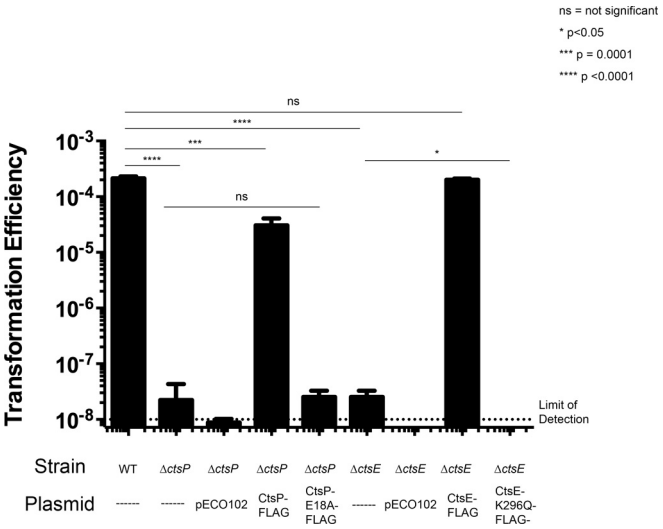

FIG 1 Transformation efficiency of *C. jejuni* CtsE and CtsP mutants. Strains are complemented with pECO102, pECO102 expressing the wild-type coding sequence with a C-terminal FLAG tag, or pECO102 expressing the coding sequence with a Walker box mutation and a C-terminal FLAG tag. The data represent averages of the results from three samples per strain from one experiment. Experiments were repeated at least three times with similar results. Error bars indicate standard deviations. The limit of detection is indicated with a dashed line. Stars indicate *P* values calculated using one-way analysis of variance (ANOVA) with Dunnett's multiple-comparison test.

While the FLAG-tagged version of CtsE could complement a CtsE deletion mutant, the Walker box A point mutant in CtsE (CtsE-FLAG K296Q) could not (Fig. 1). Immunoblotting confirmed that CtsE-FLAG K296Q was expressed at levels similar to CtsE-FLAG levels (data not shown).

Several alleles of the gene encoding CtsP-FLAG with alterations to the same Walker box A lysine proved unstable (data not shown), preventing functional assessment of Walker box A in CtsP; we thus targeted Walker box B in CtsP for mutagenesis. The conserved sequence of Walker box B is hhhhDEXX, where h is a hydrophobic amino acid and X is any amino acid. The coding sequence for the conserved glutamic acid residue (E81) was changed by site-directed mutagenesis to encode an alanine residue in CtsP-FLAG, which was stable (by immunoblotting; data not shown). CtsP-FLAG E81A was unable to complement a Δ*ctsP* mutant (Fig. 1). Through these site-directed mutagenesis studies, we have determined that intact nucleotide binding motifs are required for the function of both CtsE and CtsP in transformation.

Localization of CtsE and CtsP. Members of the PulE-VirB11 superfamily of NTPases are hypothesized to act at the inner membrane of Gram-negative bacteria through association with membrane-localized proteins within their respective transport systems. To assess localization of CtsP-FLAG and CtsE-FLAG, *C. jejuni* strains expressing either fusion protein were fractionated into soluble and insoluble fractions as described previously (34). The soluble fraction contains the cytoplasmic and periplasmic contents, and the insoluble fraction contains the inner and outer membranes. Activity of isocitrate dehydrogenase, a cytoplasmic enzyme, was monitored to determine the purity of the fractions (34). Immunoblot analysis of these fractions using anti-FLAG monoclonal antibody revealed different subcellular locations for these two putative NTPases. CtsP-FLAG was detected primarily in the

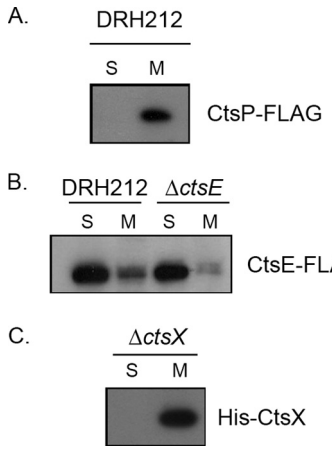

FIG 2 Localization of CtsE, CtsP, and CtsX fusion proteins. Cellular fractions of DRH212 expressing CtsP-FLAG (A), CtsE-FLAG (B), or His-CtsX (C) from pECO102 or the Δ*ctsE* mutant expressing CtsE-FLAG (B) were separated by 12% or 15% SDS-PAGE. The presence of the CtsP-FLAG and CtsE-FLAG fusion proteins was detected by immunoblotting using a monoclonal antibody against FLAG. His-CtsX was detected using a monoclonal antibody against His. S, soluble fraction; M, membrane fraction.

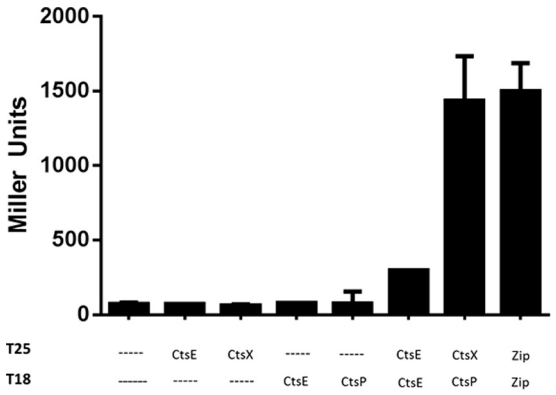

FIG 3 β-Galactosidase assays of bacterial two-hybrid-system interactions. When fused to proteins that interact, the T25 and T18 fragments of adenylate cyclase are able to associate, leading to cyclic AMP (cAMP) synthesis and resulting in transcription of *lacZ*. For a positive control, T25 and T18 were fused to a leucine zipper (Zip) (31). Activities represent averages of the results from three samples from one experiment which was repeated at least three times with similar results. Error bars indicate standard deviations.

membrane fraction, while CtsE-FLAG was located primarily in the soluble fraction (Fig. 2A and B). To determine whether the cytoplasmic localization of CtsE-FLAG resulted from competition for membrane binding partners with the chromosomally encoded version of CtsE, we assessed localization of CtsE-FLAG in a strain Δ*ctsE* background. The tagged protein was still observed primarily in the cytoplasmic fraction in the absence of native CtsE (Fig. 2B).

Bacterial two-hybrid screen for identification of Cts interaction partners. CtsP localizes to the membrane fraction of *C. jejuni* but does not have a predicted hydrophobic region that could serve as a transmembrane domain. We hypothesized that interaction with another protein might be responsible for the membrane localization of CtsP. To explore this, we used a bacterial two-hybrid system based on functional reconstitution of adenylate cyclase activity (31). For two-hybrid analysis, we tested competence genes *ctsE*, *ctsX*, *ctsF*, *ctsD*, and *ctsR*, all of which are encoded contiguously with *ctsP*, and *ctsG*, *ctsW*, and *dprA*, which are not linked to *ctsP*. Genes were cloned into both pT18 and pT25, two plasmids that provide different domains of adenylate cyclase. We tested for interactions between all Cts proteins. Two different protein-protein interactions among Cts proteins were identified (Fig. 3). One was between CtsE-T18 and T25-CtsE, which is consistent with observations that GspE proteins in other type II transport systems form multimers (26, 41, 42), suggesting that CtsE may form a multimonomer complex.

The other interaction we identified was between CtsP-T18 and T25-CtsX (Fig. 3); *ctsX* is encoded immediately downstream of *ctsP* (8). Based on Kyte-Doolittle hydropathy analysis, CtsX is predicted to be a membrane protein by virtue of a single putative transmembrane helix from amino acid 21 to amino acid 40. To test this experimentally, we expressed a His-CtsX fusion protein in *C. jejuni* DRH212. Cells were fractionated into soluble and membrane fractions as discussed above for CtsE and CtsP, and fraction purity was assessed by isocitrate dehydrogenase assays (data not shown). His-CtsX was detected by immunoblotting with an anti-His monoclonal antibody. As predicted, the majority of the His-CtsX fusion protein was located in the membrane fraction (Fig. 2C).

The membrane topology of CtsX was experimentally determined using β-galactosidase and alkaline phosphatase fusions to the CtsX coding sequence. Alkaline phosphatase is active only when transported outside the cytoplasm (35), whereas β-galactosidase is active in the cytoplasm. Portions of the CtsX coding sequence at different positions on both sides of the predicted transmembrane region of CtsX were fused in frame with *lacZ* and *phoA* reporter genes in pTrcLacZ and pTrcPhoA (32) (Fig. 4).

The first 14 amino acids of CtsX directed high-level β-galactosidase activity but low-level alkaline phosphatase activity, indicating that the N terminus of the protein resides in the cytoplasm (Fig. 4). In contrast, fragments of CtsX, including the putative transmembrane domain, directed low β-galactosidase levels but high alkaline phosphatase activity levels (Fig. 4), consistent with there being a transmembrane domain in the protein as predicted by the Kyte-Doolittle plot. Based on these data, we predict that small portion of the N terminus of CtsX is localized to the cytosol, with the remainder of the protein within the periplasmic space.

Localization of CtsP in the absence of other Cts proteins. Because CtsP lacks an obvious membrane localization signal and interacts with CtsX, which is membrane localized, we hypothesized that membrane localization of CtsP depends on CtsX. However, CtsP-FLAG localized to the membrane fraction in mutant cells lacking *ctsX* (Fig. 5A), as well as in mutants lacking other transformation genes, including *ctsF*, *ctsE*, *ctsD*, *ctsR*, and *ceuB* (8) (Fig. 5B and data not shown). To test whether membrane localization of CtsP requires any *C. jejuni* proteins, we expressed CtsP-FLAG in *E. coli* JM101. Membrane localization was still observed (Fig. 5C), suggesting either that membrane localization is an intrinsic feature of CtsP or that a protein with which it interacts and colocalizes is also present in *E. coli*.

To rule out the possibility that the overexpressed protein is simply insoluble and thus pellets with membranes in the fractionation experiments described above, we tested membrane association of CtsP using a membrane flotation assay. Membrane flotation assays utilize a sucrose gradient to separate the membranes

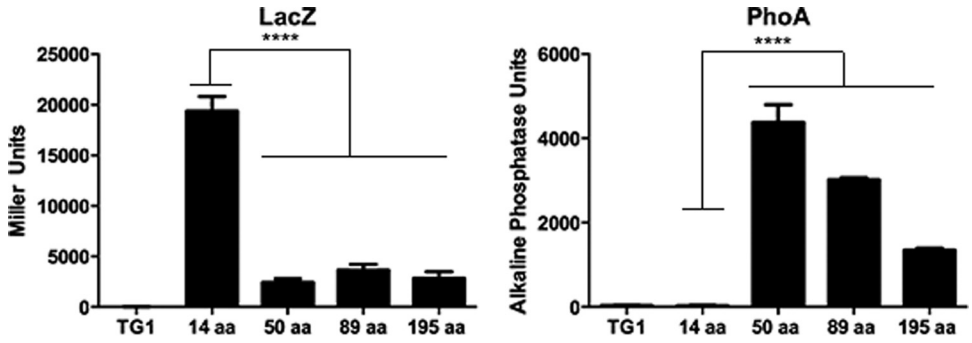

FIG 4 Topology studies of CtsX. Reporter protein activity levels of *E. coli* TG1 expressing four fragments of *ctsX* in a translational fusion with *lacZ* (left) or with *phoA* (right). Data represent averages of the results from three samples from one experiment that was repeated at least three times with similar results. Error bars indicate standard deviations. Stars represent *P* values calculated using Student's unpaired *t* test.

from any insoluble proteins that would pellet during ultracentrifugation. Using this assay, we observed that, while some CtsP did become insoluble (in fraction 6), the majority was located in membrane-containing fractions (fractions 2 to 5). This was true when CtsP-FLAG was expressed in wild-type cells as well as in the Δ*ctsP*, Δ*ctsX*, or Δ*ctsE* strains. Even when CtsP-FLAG was expressed in *E. coli*, it was primarily localized to the membrane-containing fraction and not the insoluble fraction (Fig. 6).

Membrane extractability of CtsP and CtsX. Given its fractionation with the membrane of both *C. jejuni* and *E. coli*, we hypothesized that CtsP is a peripheral membrane protein, perhaps associating with the membrane by interacting with the polar head of the phospholipid bilayer. In contrast, CtsX, with its more obvious transmembrane domain and the fusion protein's ability to localize alkaline phosphatase to the periplasmic space (Fig. 4), appears to be an integral membrane protein. To further characterize CtsP and CtsX as integral or peripheral membrane proteins, we analyzed the avidity of their association with *C. jejuni* membranes by employing different extraction procedures commonly used to release peripherally associated membrane proteins (43).

Membranes of *C. jejuni* expressing CtsP-FLAG or His-CtsX were treated with high concentrations of salt (1 and 2 M NaCl, 1 M KCl) or urea (3 M, 5 M) or extremes of pH (Na_2CO_3 at pH 3.0 or pH 11.0) for 30 min at 4°C as described in Materials and Methods. Control membranes were treated with 10 mM Tris (pH 8.0). CtsP-FLAG was partially extracted from the membranes in the presence of high salt concentrations (1 and 2 M NaCl, 1 M KCl), while CtsX remained predominantly membrane associated (Fig. 7). Treatment of the membranes with urea (3 M, 5 M) nearly completely solubilized CtsP-FLAG, while His-CtsX was extracted only partially by this treatment (Fig. 7). Membrane treatment with 0.5 M Na_2CO_3 at pH 11 also extracted CtsP-FLAG into the soluble fraction (Fig. 7). In contrast, Na_2CO_3 treatment was not sufficient to

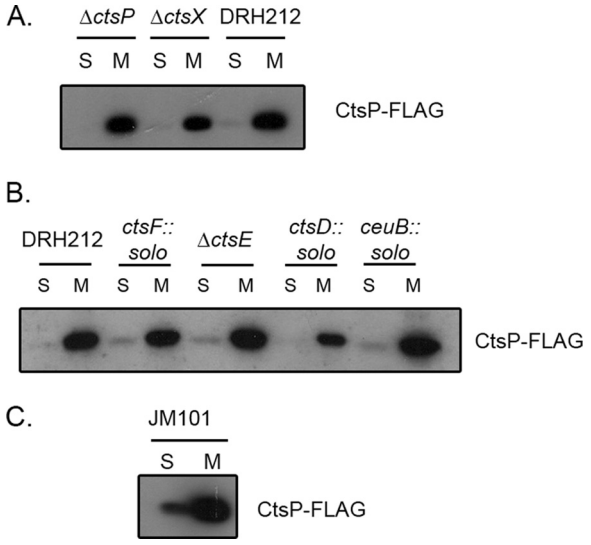

FIG 5 Localization of CtsP in the absence of other Cts proteins. Localization of CtsP-FLAG is indicated. Cellular fractions of DRH212 or *cts* mutants (A or B) or cellular fractions from *E. coli* JM101 expressing CtsP-FLAG from pECO102 (C) were separated by 12% SDS-PAGE. The presence of the CtsP-FLAG fusion protein was detected by immunoblotting using a monoclonal antibody against the FLAG epitope. S, soluble fraction; M, membrane fraction.

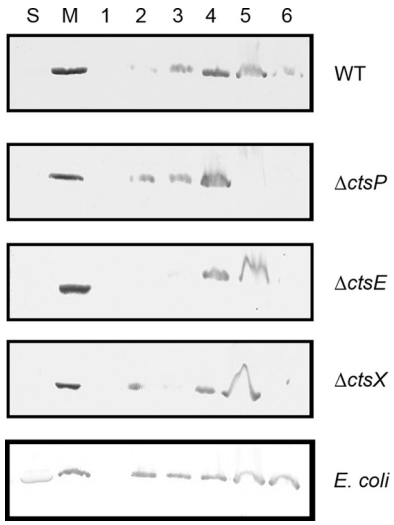

FIG 6 Membrane flotation of CtsP-FLAG. Soluble and membrane fractions of DRH212, *cts* mutants (Δ*ctsP*, Δ*ctsE*, and Δ*ctsX*), and *E. coli* containing the fusion protein CtsP-FLAG expressed on pECO102 were obtained. The membrane fractions were resuspended in a sucrose gradient to separate the membranes from the insoluble material and subjected to ultracentrifugation, and fractions were taken moving sequentially down the gradient (numbered 1 to 6, where 1 is the top of the gradient and 6 contains the insoluble proteins). These fractions were separated via 12% SDS-PAGE. The presence of the CtsP-FLAG fusion protein was detected by immunoblotting using a monoclonal antibody against the FLAG epitope. S, soluble fraction; M, membrane fraction.

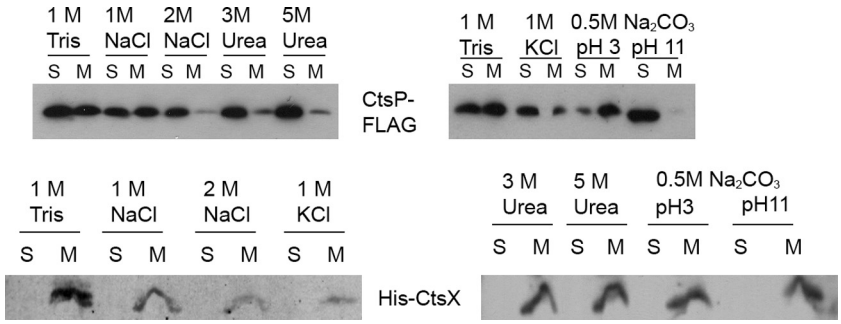

FIG 7 Membrane extractability of CtsP-FLAG and His-CtsX. Membranes were incubated with the indicated reagents for 30 min at 4°C and sedimented by ultracentrifugation. Separated fractions were examined by 12% or 15% SDS-PAGE. Fusion proteins were detected by immunoblotting with anti-FLAG M2 antibody for CtsP-FLAG and with an anti-6×His antibody for His-CtsX. S, soluble fraction after washes; M, membrane fraction.

affect His-CtsX localization, and it remained in the membrane fraction after the treatment (Fig. 7). These results suggest that while His-CtsX is an integral membrane protein, the more ready extraction of CtsP-FLAG suggests that it is not integral but is peripheral to the membrane, interacting with it perhaps through an as-yet-undefined mechanism.

DISCUSSION

The goal of this study was to characterize two putative NTPases/NTP binding proteins involved in natural transformation of *C. jejuni*. One of these, CtsE, belongs to a family of well-studied NTPases involved in transport of macromolecules in a number of systems, including type II secretion, type IV secretion, and type IV pilus biogenesis (15, 16). Members of this family have ATPase activity *in vitro* (22–26), although the exact ATP-dependent step in transport is unclear. Given the homology of CtsE to other members of this family and the importance of an intact Walker box A for CtsE to function in natural transformation, it is likely that CtsE has ATPase activity critical for its role in transformation.

The other putative NTPase/NTP binding protein, CtsP, has little homology to other NTPases and cannot be assigned to a defined family. CtsP Walker box A mutants were all unstable in *C. jejuni*, but a stable mutant protein lacking Walker box B was unable to correct the transformation defect of a Δ*ctsP* mutant. We suggest that CtsP may bind and hydrolyze nucleotides and that this property is necessary for natural transformation of *C. jejuni*, although further work is needed to determine whether CtsP has ATPase activity.

Transformation in *C. jejuni* involves a number of proteins with similarity to those required for pilus biogenesis and natural transformation in *Neisseria gonorrhoeae* and *Vibrio cholerae* (8, 44–48). In these species, transformation is facilitated by the production of a pilus that is produced and retracted through the power of two ATPases. Transformation in *N. gonorrhoeae* requires the activity of two ATPases, PilF and PilT (44, 49), and in *V. cholerae* it requires PilB and PilT (47). Both *N. gonorrhoeae* ATPases are members of the PulE-VirB11 superfamily (15, 16). PilF is required for elaboration of the pilus, while PilT is required for retraction (44, 50). As noted, while CtsE appears to fall into this family, CtsP does not. However, unlike these two systems, *C. jejuni* has never been shown to produce a pilus (8) and lacks a homolog to the major structural subunits found in these systems, PilE in *N. gonorrhoeae* and PilA in *V. cholerae*. Instead, *C. jejuni* contains a number of genes that may encode pseudopilins, *ctsG*, *ctsT*, and *Cjj81176_1096*; two of these have been shown to function in transformation (8). Whether a pilus or pseudopilus is responsible for DNA binding or uptake across the outer membrane, requiring the actions of CtsE and CtsP, has yet to be determined. In the *V. cholerae* transformation system, pseudopilins are thought to initiate pilus formation whereas the major pilin subunit, PilA, is then responsible for the pilus structure (47). Loss of any of these proteins leads to an approximately 3-log decrease in transformation efficiency (47). Similarly to the *V. cholerae* system, the pseudopilins in *C. jejuni* may be sufficient to induce transformation. A second possibility is that, unlike the process in the *V. cholerae* system, the pseudopilins also form enough of a pilus-like structure to facilitate transformation even without the presence of a major pilin subunit.

CtsE appears to be predominantly cytosolic in *C. jejuni*, whereas CtsP is predominantly membrane associated. A CtsE homologue, ComGA, involved in transformation of *Bacillus subtilis* localizes to the membrane in that species, where it behaves as a peripheral membrane protein (51). In some other bacteria, CtsE homologues (generally termed GspE proteins) become membrane localized by interacting with an integral membrane protein generally termed GspL (52, 53) but no GspL homologue was identified in the *C. jejuni* genome sequence. It is difficult to imagine how CtsE could play a role in the early stages of transformation unless it interacts with proteins at the membrane. In the *Vibrio cholerae* system, a green fluorescent protein (GFP)-tagged version of the CtsE homolog, PilB, forms dynamic foci at the membrane that transiently overlap other components of the *Vibrio* transformation apparatus (47). Perhaps similarly to the *Vibrio* system, a transient, or very weak, association of CtsE occurs with the membrane and this could have been disrupted during fractionation; this might account for the small amount of CtsE-FLAG observed in the membrane fraction.

Unlike CtsE, CtsP localizes to the membrane in *C. jejuni*, where it may interact with other components of the transformation machinery. By bacterial two-hybrid analysis, we detected an interaction between CtsP and CtsX, another protein necessary for efficient *C. jejuni* transformation. CtsX is encoded immediately downstream of CtsP in a putative operon (8). CtsX is an integral membrane protein with its amino terminus exposed to the cytoplasm and to residues 50 to 195 in the periplasm. Interaction with CtsX is not necessary for CtsP localization to the membrane; neither is interaction with several other components of the transformation machinery. CtsP behaves as a peripheral membrane pro-

tein, and it may associate directly with the membrane. This possibility is strengthened by the membrane localization of CtsP-FLAG observed during expression in *E. coli*. If membrane localization required interaction with another protein, it would have to be present in *E. coli* as well and would likely not be involved in natural transformation, a process that has not been described in *E. coli*. As noted above, type II secretion system GspE family members, with which CtsE shares homology, associate with the membrane through interaction with another protein, GspL. When these GspE family members are expressed in *E. coli*, cytoplasmic localization is observed (54), which differs from our observed localization of CtsP to membrane fractions in *E. coli*. Further study is needed to confirm that CtsP directly associates with the membrane. Furthermore, we were able to replicate the membrane association phenotype using membrane flotation. If this association were an artifact of the initial fractionation procedure, we would expect to see CtsP-FLAG associate predominately with the insoluble aggregate and not float with the membranes. One possibility that has yet to be discounted definitively is that the CtsP-FLAG localization (as well as that of CtsE-FLAG) is due to studying plasmid-encoded proteins rather than the chromosomally encoded native protein. But the behavior of the plasmid-encoded, FLAG-tagged proteins mitigates this concern, because both CtsE-FLAG and CtsP-FLAG restore the transformation efficiency of their respective mutant strains to near-wild-type levels.

CtsX and CtsP represent novel components of the *C. jejuni* transformation machinery. Homologues of CtsX have not been identified in other transformation systems, and BLAST analysis does not provide obvious clues about its function. As it resides largely in the periplasm, perhaps its C terminus interacts with other components of the transformation machinery in that compartment. CtsX and CtsP are encoded in a putative operon between the type II secretion/type IV pilus biogenesis system homologues *ctsD*, *ctsE*, and *ctsF*. Given the CtsX/CtsP interaction, we hypothesize that these two proteins comprise a component of type II secretion/type IV pilus biogenesis systems specifically involved in *C. jejuni* competence. Perhaps the interaction is required for assembly or translocation of other transformation components or allows assembly of a structure needed specifically for DNA uptake and not pilus biogenesis.

ACKNOWLEDGMENTS

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