Conjugative Coupling Proteins and the Role of Their Domains in Conjugation, Secondary Structure and in vivo Subcellular Location

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Type IV Coupling Proteins (T4CPs) are essential elements in many type IV secretion systems (T4SSs). The members of this family display sequence, length, and domain architecture heterogeneity, being the conserved Nucleotide-Binding Domain the motif that defines them. In addition, most T4CPs contain a Transmembrane Domain (TMD) in the amino end and an All-Alpha Domain facing the cytoplasm. Additionally, a few T4CPs present a variable domain at the carboxyl end. The structural paradigm of this family is TrwB\textsubscript{R388}, the T4CP of conjugative plasmid R388. This protein has been widely studied, in particular the role of the TMD on the different characteristics of TrwB\textsubscript{R388}. To gain knowledge about T4CPs and their TMD, in this work a chimeric protein containing the TMD of TraJ\textsubscript{pKM101} and the cytosolic domain of TrwB\textsubscript{R388} has been constructed. Additionally, one of the few T4CPs of mobilizable plasmids, MobBC\textsubscript{CloDF13} of mobilizable plasmid CloDF13, together with its TMD-less mutant MobBC\textsubscript{ΔTMD} have been studied. Mating studies showed that the chimeric protein is functional in vivo and that it exerted negative dominance against the native proteins TrwB\textsubscript{R388} and TraJ\textsubscript{pKM101}. Also, it was observed that the TMD of MobBC\textsubscript{CloDF13} is essential for the mobilization of CloDF13 plasmid. Analysis of the secondary structure components showed that the presence of a heterologous TMD alters the structure of the cytosolic domain in the chimeric protein. On the contrary, the absence of the TMD in MobBC\textsubscript{CloDF13} does not affect the secondary structure of its cytosolic domain. Subcellular localization studies showed that T4CPs have a unipolar or bipolar location, which is enhanced by the presence of the remaining proteins of the conjugative system. Unlike what has been described for TrwB\textsubscript{R388}, the TMD is not an essential element for the polar location of MobBC\textsubscript{CloDF13}. The main conclusion is that the characteristics described for the paradigmatic TrwB\textsubscript{R388} T4CP should not be ascribed to the whole T4CP family. Specifically, it has been proven that the mobilizable plasmid-related MobBC\textsubscript{CloDF13} presents different characteristics regarding the role of its TMD. This work will contribute to better understand the T4CP family, a key element in bacterial conjugation, the main mechanism responsible for antibiotic resistance spread.

Keywords: coupling proteins, type IV secretion systems, bacterial conjugation, membrane proteins, antibiotic resistance spread
INTRODUCTION

Type IV coupling proteins (T4CPs) are essential elements in the conjugative type IV secretion systems (T4SSs) and are also key elements in many pathogenic T4SSs. The members of this family display a high sequence, length, and domain architecture heterogeneity being the Nucleotide-Binding Domain (NBD) the only conserved domain in all T4CPs. For this reason, they are classified according to the different domain architectures: (i) VirD4-type subfamily that are integral membrane proteins; (ii) TraG-J pairs, which are also integral membrane proteins but additionally present a physical and functional association with another membrane protein of the T4SS; (iii) T4CPs without Transmembrane Domain (TMD), which could or could not interact with other T4S membrane proteins creating a VirD4-type complex, like the pair TraIP501 and TraIP501; (iv) FtsK-like T4CPs; and (v) Archaeal T4CPs.

The structural paradigm of this family is the T4CP of conjugative plasmid R388, TrwB. It is a VirD4-type protein composed of a TMD at the N-terminus (consisting of two transmembrane α-helices connected through a small periplasmic loop) and a bulky globular cytosolic domain (CD). TrwBR388 is the only full-length T4CP that has been successfully purified to date (Hormaeche et al., 2002; Redzej et al., 2017), while trials for purifying other membrane T4CPs have not rendered the sufficient amounts of high quality protein for performing in vitro assays (Chen et al., 2008). For this reason most of the in vitro studies of T4CPs have been achieved using deletion mutant proteins that lack the TMD (Schroder and Langa, 2003; Tato et al., 2007; Larrea et al., 2017). In this regard, the TMD deletion mutant protein of TrwBR388, TrwBΔN70, was resolved by X-ray crystallography, showing that the CD of TrwBR388 contains an NBD with the Walker A and Walker B motifs and a small membrane-distal All-Alpha Domain (AAD) (Gomis-Rüth et al., 2001).

Comparative studies of the properties of TrwBR388 and TrwBΔN70 showed significant differences regarding biological activity (such as in vivo function, in vitro nucleotide-binding properties, and in vitro ATPase activity), oligomerization pattern, subcellular location, and stability (Moncalián et al., 1999; Vecino et al., 2010, 2011; Hormaeche et al., 2002, 2004, 2006; Segura et al., 2013, 2014). For this reason it has been concluded that the TMD of TrwBR388 accomplishes a role beyond the anchorage of the protein to the membrane, influencing the location, stability, and activity of this protein.

To delve into the role of the TMD in T4CPs two different strategies have been followed. On the one hand, we have constructed a chimeric protein named TMD

\[ \text{TMD} \]

\[ \text{TrwB} \]

composed of the TMD of TraIPKM101, the phylogenetically closest T4CP to TrwBR388 from the conjugative plasmid pKM101 (Paterson et al., 1999; Alvarez-Martinez and Christie, 2009) and the CD of TrwBR388. This strategy has already been used for the study of components of T4SSs, showing interesting results (Bourg et al., 2009). Specifically, through a chimeric protein approach the function of the AAD of VirD4A1 from the T-plasmid of Agrobacterium tumefaciens (Whitaker et al., 2016) and of the N-terminal HUH domain of TrwC (Agúndez et al., 2018) have been analyzed. On the other hand, the T4CP from the mobilizable plasmid CloDF13, MobBCloDF13 and its deletion protein lacking the TMD, MobBATMD, have been constructed and studied. It is an interesting system to characterize since it is part of the rare MOB_C1 plasmid family, which are mobilizable plasmids that encode their T4CP (Smillie et al., 2010). Additionally, MobBCloDF13 has been described as an atypical T4CP, due to its dual role in DNA transfer, since it acts as an accessory protein in CloDF13 relaxation process and also as a T4CP (Nuñez and de la Cruz, 2001).

We studied the functionality of these proteins in plasmid transfer, secondary structure, thermal stability, and subcellular localization. Our findings indicate that the TMD plays different roles in conjugative plasmid related and mobilizable plasmid related T4CPs. Specifically while the TMD could play a regulatory role in TrwBR388 this cannot be inferred from the results about MobBCloDF13.

MATERIALS AND METHODS

Materials

n-dodecyl β-D maltoside (DDM) was purchased from Anatrace (Santa Clara, CA, United States). Mouse anti-His (C-term) monoclonal antibody and Alexa Fluor goat anti-mouse antibody were purchased from Invitrogen (Carlsbad, CA, United States) and Molecular Probes (Eugene, OR, United States), respectively. All buffers employed in this work are shown in Supplementary Table S1.

Bacterial Strains and Bacterial Growth Conditions

E. coli DH5α strain was used as host for plasmid constructions. This strain also served as the donor for mating experiments by hosting the plasmids of interest. E. coli UB1637 served as the recipient for mating experiments. E. coli Lemo21 (DE3), E. coli BL21 (DE3), and E. coli BL21C41 (DE3) strains were used for protein production, purification, and in vivo localization.

E. coli strains were grown in LB medium and when necessary antibiotics were added at the following final concentrations: ampicillin (100 µg/mL), streptomycin (50 µg/mL), kanamycin (50 µg/mL), chloramphenicol (12.5–25 µg/mL), and thrimethoprim (10 µg/mL).

Plasmids

The plasmids and oligonucleotides used in this study are listed in Tables 1, 2, respectively.

pSU4814 and pSU4833 plasmids were kindly provided by Fernando de la Cruz. pOPINE (Addgene plasmid # 26043; RRID: Addgene_26043) and pOPINE-3C-eGFP (Addgene plasmid # 41125; RRID: Addgene_41125) plasmids were a gift from Ray Owens. pKM101Sp^−_ΔtraI was kindly provided by Peter J. Christie. pKM101Amp^−_ΔtraI plasmid was obtained by cleavage

\[ \text{http://n2t.net/addgene:26043} \]
\[ \text{http://n2t.net/addgene:41125} \]
of the *spc* cassette of pKM101Spc ΔatrJ plasmid using *EcoRI* restriction enzyme.

**Cloning of T4CPs**

To construct the chimeric protein TMD<sub>TraJ</sub>CD<sub>TrwB</sub> and the transmembrane deletion mutant protein of MobB<sub>CloDF13</sub>, MobB ΔTMD, the sequences of TrwB<sub>R388</sub>, TraJ<sub>PKM101</sub>, and MobB<sub>CloDF13</sub> were analyzed through bioinformatics tools. First, the different characteristics of the proteins, such as molecular weight and isolectric point, were analyzed using ProtParam.<sup>3</sup> Second, the topology of the membrane proteins was studied using Topcons<sup>4</sup> (Tsirigos et al., 2015).

### TABLE 1 | Plasmids employed in this work.

| Plasmid         | Description                        | Phenotype                  | References            |
|-----------------|------------------------------------|----------------------------|-----------------------|
| pET24a (+)      | Expression vector                  | Kan<sup>†</sup>, C-terminal His tag | Novagen               |
| R388            | Natural plasmid                    | Tmp<sup>†</sup>, TRA<sub>W</sub>, IncW   | Datta and Hedges, 1972 |
| pKM101          | Natural plasmid                    | Amp<sup>†</sup>, TRA<sub>W</sub>, IncN | Langer and Walker, 1981 |
| pKM101ΔatrJ     | pKM101ΔatrJ and Sp<sup>R</sup> resistance cassette | Amp<sup>†</sup>, TRA<sub>W</sub>, IncN, TraJ<sup>−</sup> | This work            |
| pOPINE-3C-eGFP  | Expression vector                  | TraJTMD-trwBCD-GFP          | Whitaker et al., 2016 |
| pOPINE-3C-eGFP-mobB | pOPINE-3C-eGFP::mobB | pOPINE-3C-eGFP::MobCD<sub>13</sub>GFP expression under T7 promoter | This work             |
| pUBQ4 (K142T)   | pUBQ4 (K142T)                      | Ampr<sup>†</sup>             | Berrow et al., 2007   |
| pET24a (+)      | pET24a (K142T)                     | Amp<sup>†</sup>, C-terminal His tag | OPPF-UK (Addgene)     |
| pSU1456         | R388: Δatr<sub>B</sub>            | Su<sup>†</sup>, Tmp<sup>†</sup>, TRA<sub>W</sub>, IncW, TrwB<sup>−</sup> | Llosa et al., 1994    |
| pSU4814         | pSU14::mobCD<sub>13</sub>         | Chf<sup>†</sup>, Rep (p15A), Mob (CloDF13) | Nuñez and de la Cruz, 2001 |
| pSU4833         | pSU4814::mob<sub>B</sub>          | Chf<sup>†</sup>, Rep (p15A), MobB<sup>−</sup> | Nuñez and de la Cruz, 2001 |
| pUB9            | pWaldo-GFPe-trwB-GFP               | Kan<sup>†</sup>, TrwB-TEV-GFP-H<sub>B</sub>, expression under T7 promoter | Segura et al., 2014   |
| pUBQ4           | pET24a (+):traJTMD-trwBCD          | Kan<sup>†</sup>, TMD<sub>TraJ</sub>CD<sub>TrwB</sub>-H<sub>B</sub>, expression under T7 promoter | This work             |
| pUBQ4 (K142T)   | pET24a (+):traJTMD-trwBCD (K142T) | Kan<sup>†</sup>, TMD<sub>TraJ</sub>CD<sub>TrwB</sub>-H<sub>B</sub>, containing the K142T mutation in the CD<sub>TrwB</sub> domain, expression under T7 promoter | This work             |
| pUBQ4GFP        | pWaldo-GFPe-traJTMD-trwBCD-GFP     | Kan<sup>†</sup>, TMD<sub>TraJ</sub>CD<sub>TrwB</sub>-TEV-GFP-H<sub>B</sub>, expression under T7 promoter | This work             |
| pWaldo-GFPe     | Expression vector                  | Kan<sup>†</sup>, C-Terminal GFP and His tag | Waldo et al., 1999    |

### TABLE 2 | Oligonucleotides used in this work.

| Construct       | Template       | Protein          | Sequence (5′ → 3′) | Cloning technology |
|-----------------|----------------|------------------|--------------------|--------------------|
| pOPINE-mobB     | pSU4814        | MobB<sub>CloDF13</sub> | F: ACGAGATATACCATGGTTTAATACGGATTTCGGTGTGGCGTGGCAAGTTG | In-fusion<sup>a</sup> |
| pOPINE-MobBΔTMD | pSU4814        | MobBΔTMD         | R: GTGATGGTGATGTGGTGTACAGCTACGCCGCGCGCGCGCGCAACCGCGCGCG | In-fusion<sup>b</sup> |
| pOPINE-3C-eGFP- mobB | pSU4814 | MobB<sub>CloDF13</sub>GFP | F: AGGAGATATACCATGGTTTAATACGGATTTCGGTGTGGCGTGGCAAGTTG | In-fusion<sup>3</sup> |
| pOPINE-3C-eGFP- MobBΔTMD | pSU4814 | MobBΔTMDGFp | R: GTGATGGTGATGTGGTGTACAGCTACGCCGCGCGCGCGCGCAACCGCGCGCG | In-fusion<sup>3</sup> |
| pUBQ4 (K142T)   | pUBQ4          | TMD<sub>TraJ</sub>CD<sub>TrwB</sub> (K142T) | F: CAGAACTTCCAGGGCTGCCTCGTGGCCTGCGTGGCAGTTG | Site-Directed mutagenesis<sup>b</sup> |
| pUBQ4GFP        | pUBQ4          | TMD<sub>TraJ</sub>CD<sub>TrwB</sub>GFp | R: CGCGGTTCGAGATGAGCGTAAAAAGAGG | Restriction enzymes (Khol and BamHI) |

<sup>a</sup>Berrow et al. (2007).<sup>b</sup>QuickChange II Site-Directed Mutagenesis (Stratagene, San Diego, CA, United States).

<sup>3</sup>http://topcons.cbr.su.se/

<sup>4</sup>https://web.expasy.org/protparam/
Then different constructions were achieved as follows:

**TMD<sub>TraJ</sub>CD<sub>TraJ</sub>** chimeric protein consists of amino acids M<sub>1</sub>-D<sub>76</sub> from Tra<sub>JKM101</sub> followed by amino acids L<sub>71</sub>-I<sub>507</sub> from Trw<sub>B</sub><sub>388</sub>. The *tmd<sub>TraJ</sub>cd<sub>TraJ</sub>* sequence was synthesized *de novo* and inserted into pET24a (+) plasmid vector using *NdeI* and *XhoI* restriction sites, rendering pUBQ4 plasmid to produce the chimeric protein. This construction was made by TOP Gene Technologies, Inc. (Saint-Lauren, QC-Canada). To study the role of the conserved lysine of the Walker A motif (K<sub>142</sub>), this residue was substituted with a threonine using the QuickChange II Site-Directed Mutagenesis Kit from Stratagene.

To clone the plasmid vector using *XhoI* and *XbaI* restriction sites, rendering pUBQ4 plasmid to produce the chimeric protein. This construction was made by TOP Gene Technologies, Inc. (Saint-Lauren, QC-Canada). To study the role of the conserved lysine of the Walker A motif (K<sub>142</sub>), this residue was substituted with a threonine using the QuickChange II Site-Directed Mutagenesis Kit from Stratagene (San Diego, CA, United States) to obtain the TMD<sub>TraJ</sub>CD<sub>TraJ</sub> (K142T) protein. Additionally, to clone the *tmd<sub>TraJ</sub>cd<sub>TraJ</sub>-eGFP* gene *tmd<sub>TraJ</sub>cd<sub>TraJ</sub>* sequence was inserted into the pWaldo-GFP<sub>e</sub> plasmid vector using *XhoI* and *BamHI* restriction sites (Segura et al., 2014).

To clone Mob<sub>B</sub>CD<sub>DF13</sub>-related proteins, the cloning of *mobB*, *MobBA<sup>TM</sup>D*, and *MobB<sup>ΔTM</sup>D<sup>-eGFP</sup>* genes was performed in the Oxford Protein Production Facility (OPPF-UK) using the High-throughput protocol described by Bird (2011). Specifically, Mob<sub>B</sub>ΔTM<sup>1</sup> soluble mutant protein consists of amino acids D<sub>185</sub>-Y<sub>55</sub> of Mob<sub>B</sub>CD<sub>DF13</sub> obtained after deletion of amino acids M<sub>1</sub>-A<sub>184</sub> from wild type Mob<sub>B</sub>CD<sub>DF13</sub>. All the oligonucleotides employed in the aforementioned cloning experiments are specified in Table 2.

### Overexpression and Purification

The same purification protocol was followed for TMD<sub>TraJ</sub>CD<sub>TraJ</sub> and Mob<sub>B</sub>CD<sub>DF13</sub> proteins. Briefly, *E. coli* BL21C41 (DE3) cells freshly transformed with plasmids pUBQ4 for TMD<sub>TraJ</sub>CD<sub>TraJ</sub> and pOPINE-<i>mobB</i> for Mob<sub>B</sub>CD<sub>DF13</sub> were grown overnight in LB (8 flasks of 10 mL) supplemented with the corresponding antibiotics at 37°C with continuous shaking. Then, cells were diluted 1:50 (v/v) with fresh LB supplemented with antibiotics (8 flasks of 500 mL) and were grown at 37°C with continuous shaking until an OD<sub>600</sub> of 0.4ñ0.5 was achieved. Next, overexpression was induced by the addition of 1 mM isopropyl α-D-thiogalactopyranoside (IPTG) and cells were grown with continuous shaking at 25°C overnight. Cells were harvested by centrifugation at 8,000 g for 15 min at 4°C. The pellet was suspended in 80 mL of Cell buffer, frozen with liquid N<sub>2</sub> and stored at −80°C.

For purification, cells were thawed at 37°C and 0.02 mg/mL DNase I, 1 mM dithiothreitol (DTT), 0.07% (w/v) lysozyme, 1 mM MgCl<sub>2</sub>, 1 mM phenylmethanesulfonyl fluoride (PMSF) and two tablets of cOmplete<sup>TM</sup> EDTA-free Protease Inhibitor Cocktail from Sigma-Aldrich (San Luis, MO, United States) were added. From this point onward, the whole process was performed at 4°C to avoid aggregation of the proteins. After 45 min of incubation with agitation, cells were disrupted by sonication and centrifuged at 8,000 g for 15 min to remove non-lysed cell. The supernatant, containing the broken cells, was centrifuged at 138,000 g for 45 min to pellet the membrane fraction which was subsequently carefully resuspended in 30 mL of Cell buffer. Then DDM and NaCl were added to final concentrations of 19.6 mM and 600 mM, respectively, and the volume was adjusted to 40 mL. The mixture was incubated for 90 min with continuous stirring and then centrifuged at 138,000 g for 1 h.

The supernatant containing the protein to be purified was mixed 1:1 (v/v) with MP1 buffer to decrease the concentration of DDM and NaCl to 8.3 mM and 300 mM, respectively. Then, the sample was supplemented with 50 mM imidazole and loaded onto a 5 mL HisTrap<sup>TM</sup> FF (GE Life Sciences; Marlborough, MA, United States) column previously equilibrated with MP2 buffer. To increase the binding of the protein, the sample was left recirculating overnight. Next, it was connected to an ÄKTA-FPLC system and it was washed with 50 mL of MP2 buffer at a flow rate of 2 mL/min until the absorbance at 280 nm reached the baseline. Bound proteins were eluted with MP3 buffer, at a flow rate of 1.5 mL/min and fractions of 1 mL were collected. Obtained samples were analyzed by SDS-PAGE and the ones containing each target protein were pulled-down and concentrated using a centrifugal filter with a MWCO of 100 kDa. Then, 5 mL of the resulting samples were separately loaded onto a Superdex 200 HR 16/60 column and the size-exclusion chromatography (SEC) was performed in MP purification buffer at 0.5 mL/min. The fractions corresponding to each target protein were pulled-down and concentrated as explained before. Glycerol was added to a final concentration of 20% (v/v) and protein concentration was determined by measuring absorption at 280 nm. Finally, aliquots were stored at −80°C.

When TMD<sub>TraJ</sub>CD<sub>TraJ</sub> was purified with the aim of performing infrared spectroscopy (IR) assays the DDM and NaCl concentrations of the MP purification buffer were changed to the ones described in the previously published purification protocols of Trw<sub>B</sub><sub>388</sub> and Trw<sub>B</sub>Δ<sub>N50</sub> (i.e., 0.2 mM DDM and 200 mM NaCl instead of 0.6 mM DDM and 300 mM NaCl) (Vecino et al., 2011, 2012).

For MobBA<sup>TM</sup>D purification, *E. coli* Lemo21 (DE3) cells freshly transformed with pOPINE-MobBA<sup>TM</sup>D plasmid were grown in 4 L of LB supplemented with ampicillin at 37°C with continuous shaking until an OD<sub>600</sub> of 0.5ñ0.6 was reached. Expression was induced with 1 mM IPTG and performed for 20 h at 25°C. Cells were harvested and stored as explained previously.

For protein purification, the cell suspension was thawed and the lysis protocol previously described for TMD<sub>TraJ</sub>CD<sub>TraJ</sub> and Mob<sub>B</sub>CD<sub>DF13</sub> was followed. Then, the sample was centrifuged at 138,000 g for 45 min to pellet the membrane fraction and the inclusion bodies. The supernatant with the soluble proteins was supplemented with 50 mM imidazole and loaded onto a 5 mL HisTrap<sup>TM</sup> FF (GE Life Sciences; Marlborough, MA, United States) column, previously equilibrated with MobBA<sup>TM</sup>D<sub>1</sub> buffer. Affinity chromatography was performed as previously described for Mob<sub>B</sub>CD<sub>DF13</sub>, but using MobBA<sup>TM</sup>D<sub>1</sub> buffer for washing and MobBA<sup>TM</sup>D<sub>2</sub> buffer for elution. Fractions containing the target protein were pulled-down and concentrated using a Centricon YM-30 to a final volume of 600 μL. The resulting sample was centrifuged to remove aggregates and loaded onto a Superdex 200 HR 10/30 column. The SEC was performed in Cell buffer at 0.3 mL/min and 0.5 mL fractions were collected. Fractions that contained the protein of interest were pulled-down and the sample was centrifuged to discard the aggregates.
Glycerol was added to a final concentration of 20% (v/v) and aliquots were made.

**Mating Assays**

Mating assays were performed as described by Llosa et al. (2003) with small modifications. Briefly, donors (*E. coli* DH5α co-transformed with the appropriate plasmids) and recipient cells (*E. coli* UB1637) were grown in LB supplemented with the corresponding antibiotics overnight at 37°C. Each mating assay 100 µL of the donor and the recipient cells were mixed, centrifuged, resuspended in 50 µL LB and placed onto a GS Millipore filter (0.22 µm pore size) settled on a pre-warmed LB-agar plate. After 1 h incubation at 37°C bacteria were washed from the filters in 2 mL LB by shaking at 450 rpm for 20 min and vortexing for 30 s. Then, 100 µL of the appropriate dilutions were plated on selective media for donors and transconjugants. The plates were incubated overnight at 37°C and the colonies were counted, normalizing the conjugation frequency as the number of transconjugants per donor cell.

**Infrared Spectroscopy**

To accomplish IR studies of different T4CPs and their variants (i.e., TMD_TrajCD_TrwB, MobB_CloDF13, and MobB_ATMD) purification of each protein was carried out as previously described. The H-D exchange protocol was performed at 4°C and adapted for each protein. Briefly, TMD_TrajCD_TrwB was diluted with the IR buffer, dialyzed against the same buffer using a D-Tube™ Dialyzer Midi (MWCO 3.5 kDa) (Merck; Darmstadt, Germany), diluted again in IR buffer and concentrated using an Amicon Ultra-0.5 mL centrifugal filter (MWCO: 100 kDa). A similar process was followed for MobB_CloDF13 except for the dialysis step. Regarding MobB_ATMD, sample was diluted, dialyzed and concentrated as described for TMD_TrajCD_TrwB but using in MobB_ATMD IR buffer. Final protein samples were always above 1 mg/mL protein concentration.

Infrared spectra were recorded in a Thermo Nicolet Nexus 5700 (Thermo Fisher Scientific; Waltham, MA, United States) spectrometer equipped with a liquid nitrogen-refrigerated mercury-cadmium-telluride detector using a Peltier-based temperature controller (TempComp™, BioTools; Wauconda, IL, United States), and a 25 µm optical path. Typically 370 scans for each, background and sample, were collected at 2 cm⁻¹ resolution and averaged after each minute. Spectra were collected with OMNIC software (Nicolet) and data processing was performed with OMNIC and SpectraCalc, following previously resolved methods (Arrondo et al., 1993; Arrondo and Goni, 1999).

The information about the secondary structure and about the thermal denaturation of T4CPs and their derivatives was obtained by IR spectroscopy through analysis of the infrared amide I band that corresponds mainly to the C = O stretching vibrations of the peptide bonds and which is located between 1700 and 1600 cm⁻¹ region of the IR spectrum. Amide I band is conformationally sensitive and can be used to monitor the protein secondary structure composition and changes induced by thermal denaturation. Secondary structure studies were made at 20°C and band decomposition of the amide I was performed as previously reported (Vecino et al., 2011, 2012). For thermal stability studies samples were heated from 20 to 80°C at a rate of 1°C/min.

**Subcellular Location of T4CPs**

Subcellular location of different T4CPs and their variants (i.e., TrwB_R388, TMD_TrajCD_TrwB, MobB_CloDF13, and MobB_ATMD) was achieved by confocal fluorescence microscopy. To do so, two different approaches were used: (i) eGFP-labeling (Cormack et al., 1996) and (ii) immunofluorescence. Since the eGFP moiety only emits fluorescence when properly folded (Drew et al., 2005), the eGFP based approach allowed visualizing only properly folded proteins.

Prior to localization assays, the in vivo activity of the T4CP-eGFP fusion-proteins was proved by mating assays as previously described (Supplementary Table S2). Afterward, T4CP-eGFP fusion-proteins were expressed in *E. coli* BL21C41 (DE3) strain, except for MobB_ATMD that was expressed in BL21 (DE3) strain. To do so cells were transformed with pUBQ4, by induction with 1 mM IPTG at OD₆₀₀ 0.4–0.5 for the membrane proteins and OD₆₀₀ 0.5–0.6 for MobB_ATMD. Protein expression was performed for 4 and 20 h at 25°C. Additionally, the subcellular location of TrwBR388-related proteins was determined in the presence of pSU1456 plasmid, which codes for all the R388 conjugative proteins except TrwBR388. Similarly, MobB_CloDF13 was also expressed in the presence of plasmid pSU1456 and to mimic the in vivo transfer of CloDF13, its location was additionally studied in the presence of plasmids pSU1456 (R388 plasmid that lacks TrwBR388 protein) and pSU4833 (CloDF13 plasmid that contains its mobilization region except for MobB_CloDF13 protein). Sample handling was performed as described by Segura et al. (2014). The images were acquired in a Leica TCS SP5 confocal fluorescence microscope, with a 60× oil immersion objective. Sample excitation was performed with 488 nm wavelength, while fluorescence emission was measured between 505 and 525 nm. The images were analyzed using Huygens and ImageJ softwares. To ease the counting process and better distinguish the different locations the images were treated with the preset ICE filter of ImageJ software; in this manner five different locations for the T4CP-eGFP fusion-proteins were described (Supplementary Figure S1).

For immunofluorescence assays protein expression was performed as with the eGFP fusion-proteins. Sample collection and handling was performed as described by Segura et al. (2014). Cells were immunostained with mouse anti-His (C-term) monoclonal antibody as primary antibody, and Alexa Fluor goat anti-mouse as secondary antibody. Image acquisition was performed in an Olympus Fluoview™ 500 confocal fluorescence microscope at the “Analytical and high-resolution microscopy in biomedicine” facility (SGIker, UPV/EHU).

**RESULTS**

Bacterial conjugation is one of the main processes responsible for the horizontal dissemination of antibiotic resistance genes.
among bacteria. One of the essential proteins in this process is the T4CP, which is ubiquitous in all conjugative systems. Despite its importance, the only widely studied T4CP is TrwB<sub>R388</sub>. Given its central role in bacterial conjugation, detailed knowledge of the T4CP family could contribute to the development of new strategies against the spread of antibiotic resistance among bacteria.

Previously published papers have highlighted the role of the TMD on different characteristics of TrwB<sub>R388</sub>, such as plasmid conjugation (Moncalián et al., 1999), subcellular localization (Segura et al., 2014), nucleotide-binding (Hormaeche et al., 2006), hexamerization (Hormaeche et al., 2002; Matilla et al., 2010), protein stability (Hormaeche et al., 2004), interaction with other proteins of the T4SS of R388 (Segura et al., 2013), and ATP hydrolase activity (Tato et al., 2005, 2007). From all these studies it was inferred that the TMD of TrwB<sub>R388</sub> has a role beyond the mere anchorage in the membrane.

To gain more knowledge about different T4CPs, and in particular about the role of their TMD in T4CP features, in this work a TrwB<sub>R388</sub> chimeric protein that combines its CD with the TMD of its phylogenetically closest T4CP, TraJ<sub>pKM101</sub>, has been studied. Also one of the few T4CPs of mobilizable plasmids, MobB<sub>CloDF13</sub>, and its TMD deletion mutant protein, MobB<sub>A</sub><sub>TMD</sub>, have been studied. Plasmid transfer, secondary structure, thermal stability, and subcellular location studies have been carried out to shed light on the functioning of this protein family and in the role of their TMD.

**Cloning of Soluble Mutant and Chimeric Proteins**

The membrane protein topologies obtained after the bioinformatic analysis performed with Topcons software of TrwB<sub>R388</sub>, TraJ<sub>pKM101</sub>, and MobB<sub>CloDF13</sub> are shown in Figure 1A. TrwB<sub>R388</sub> and TraJ<sub>pKM101</sub> have similar size and organization of their TMDs that consist of about 70 residues and contain two α-helices connected by a small periplasmic loop. In contrast, the TMD of MobB<sub>CloDF13</sub> is larger (about 150 amino acids) and is organized into three α-helices. This information was used to design the chimeric and mutant proteins studied in this work (Figure 1B). The chimeric protein TMD<sub>Traj</sub>CD<sub>TrwB</sub> was made by combination of the TMD of the T4CP TraJ<sub>pKM101</sub> and the CD of TrwB<sub>R388</sub>. In addition, in this work the T4CP of the mobilizable plasmid CloDF13, MobB<sub>CloDF13</sub>, and its TMD deletion protein MobB<sub>A</sub><sub>TMD</sub>, were constructed (Figure 1B). The theoretical molecular weights of these proteins, necessary for their purification process, were calculated using ProtParam<sup>1</sup> bioinformatic tool. The estimated molecular weights were 58.28, 73.95, and 53.13 kDa for TMD<sub>Traj</sub>CD<sub>TrwB</sub>, MobB<sub>CloDF13</sub>, and MobB<sub>A</sub><sub>TMD</sub>, respectively. Finally, the eGFP fusion-proteins (i.e., TMD<sub>Traj</sub>CD<sub>TrwB</sub>-eGFP, MobB<sub>CloDF13</sub>-eGFP, and MobB<sub>A</sub><sub>TMD</sub>-eGFP) were constructed and since they emitted a fluorescent signal, it was deduced that they were correctly folded (Drew et al., 2006).

**Functionality and Dominance Experiments**

Through mating assays two different properties of TMD<sub>Traj</sub>CD<sub>TrwB</sub> were analyzed: (i) its capacity to complement the conjugative process in the absence of another T4CP (functionality studies) and (ii) its effect on each native conjugative system (R388 or pKM101 plasmids), being the corresponding T4CP present (TrwB<sub>R388</sub> or TraJ<sub>pKM101</sub>, respectively) (dominance studies). Results obtained in mating assays are summarized in Table 3.

Our results showed that TMD<sub>Traj</sub>CD<sub>TrwB</sub> efficiently complemented the ΔtrwB mutation in R388 transfer but to a lower rate than native R388 (0.21 vs. 1.82 × 10<sup>-4</sup> transconjugants per donor, respectively). On the contrary, TMD<sub>Traj</sub>CD<sub>TrwB</sub> was unable to complement the Δtraf mutation in pKM101 transfer (Table 3). These results are in agreement with the necessary specific interactions between the CD of the T4CP and its cognate relaxase for transfer to happen as reported previously (Cabezón et al., 1997; Hamilton et al., 2000; Ilosa et al., 2003).

It has been reported that mutation of the conserved lysine in the Walker A motif rendered a transfer deficient mutant protein TrwBK136T (Hormaeche et al., 2006). Similarly, an equivalent mutant of the soluble protein TrwBΔN70, TrwBΔN70 (K136T), lacked ATPase activity (Moncalián et al., 1999), underlining the essential role of this amino acid in the activity of TrwB<sub>R388</sub>. Here we studied the effect of the equivalent point mutation in the Walker A domain, TMD<sub>Traj</sub>CD<sub>TrwB</sub>(K142T), on the transfer capacity of chimeric protein. As expected, TMD<sub>Traj</sub>CD<sub>TrwB</sub>(K142T) was unable to complement the ΔtrwB mutation in R388 plasmid transfer (Table 3). This is in agreement with the crucial role of the K residue as it has been reported with homologous mutants in other T4CPs (Moncalián et al., 1999; Kumar and Das, 2002; Gunton et al., 2005).

Next, to accomplish dominance assays, the transfer frequencies of plasmids R388 or pKM101 in the presence of the cognate T4CP and the chimeric protein were measured. It was observed that TMD<sub>Traj</sub>CD<sub>TrwB</sub> reduced the transfer frequency of R388 or pKM101 by an order of magnitude (Table 3).

**Mobilization Experiments**

CloDF13 mobilization experiments were achieved to know whether the TMD of MobB<sub>CloDF13</sub> was essential for the mobilization of the plasmid as happens with TrwB<sub>R388</sub> or not as it has been described for TcpA<sub>pCW3</sub>, whose TMD-less mutant can perform conjugation although at a lower frequency (Parsons et al., 2007). First of all, the transfer frequency of the mobilizable region of CloDF13 (plasmid pSU4833) mediated by the T4SS of R388 was analyzed. Afterwards, the complementation experiments were performed in the presence of both pSU4833 (the mobilizable region of CloDF13 without functional MobB<sub>CloDF13</sub>) and pSU1456 plasmid (encoding for R388 conjugative system except for TrwB<sub>R388</sub>). Results obtained in mobilization assays are summarized in Table 4. It was observed that cloned MobB<sub>CloDF13</sub> was functional when the T4SS of R388 was used, in agreement with what has been previously published for TrwB<sub>R388</sub> (Hormaeche et al., 2006). Similarly, the deletion
FIGURE 1 | (A) Predicted membrane topology of TrwB<sub>R388</sub>, TraJ<sub>pKM101</sub>, and MobB<sub>CloDF13</sub> proteins. Membrane topology of the different T4CPs was predicted using Topcons software. The black lines represent the inner bacterial membrane. M1, amino-terminus; COOH, carboxy-terminus. The first and last residues of each transmembrane helix are shown indicating their position in the sequence. Proteins from R388, pKM101, and CloDF13 plasmids are shown in green, blue, and purple, respectively. (B) Schematic representation of the different T4CPs and their variants used in the present study. Proteins from R388, pKM101, and CloDF13 plasmids are shown in green, blue, and purple, respectively. The transmembrane α-helices (H) and the small periplasmic loops connecting α-helices are indicated in dark boxes and stripped boxes, respectively.

of the TMD, MobBΔTMD mutant, rendered a non-functional phenotype as it occurs with other T4CP mutants that lack the TMD, such as TrwBΔN70 and PciCΔN103 (Moncalián et al., 1999; Chen et al., 2008).

Secondary Structure of T4CPs and Their Variants
Analysis of the secondary structure components of TMD<sub>Traf</sub>CD<sub>TrwB</sub>, MobB<sub>CloDF13</sub>, and MobBΔTMD were performed by IR spectroscopy through analysis of the IR amide I band. The secondary structure of TMD<sub>Traf</sub>CD<sub>TrwB</sub> was compared to those of the native TrwB<sub>R388</sub> and its mutants TrwBΔN50 and TrwBΔN70 (Vecino et al., 2012). Figure 2A shows the original spectra and the curve-fitting decomposition corresponding to TMD<sub>Traf</sub>CD<sub>TrwB</sub> purified in the presence of detergent. Band position, percentage area, and structure assignation corresponding to the deconvolved spectrum of the amide I region are summarized in Table 5 together with those previously reported of TrwB<sub>R388</sub>, TrwBΔN50, and TrwBΔN70 (Vecino et al., 2012).

The spectrum of TMD<sub>Traf</sub>CD<sub>TrwB</sub> purified in the presence of detergent exhibited four bands related to protein structure at 1671, 1654, 1640, and 1626 cm<sup>−1</sup> (Table 5). Interpretation of the results must be done taking into account that band assignation is not always a straightforward process since its position can be altered by the environment (Arrondo and Goñi, 1999).
The component at 1654 cm$^{-1}$ was assigned to $\alpha$-helix, the bands at 1626 and 1671 cm$^{-1}$ were associated with the low and high-frequency vibrations of $\beta$-sheet, respectively, although it should be noted that the later is also assigned to $\beta$-turns. And the band at 1640 cm$^{-1}$ was assigned to flexible, non-periodic elements.

When these results were compared to previously reported ones (i.e., TrwB$\text{R}_{388}$, TrwB$\Delta$N50, and TrwB$\Delta$N70) (Vecino et al., 2012) it can be observed that the proportion of the $\alpha$-helix (35%) is lower than the one observed in the native protein TrwB$\text{R}_{388}$ purified in detergent (41%) and higher than the one shown in the deletion mutant proteins (26%). This result can be directly associated to the presence of a TMD both in TrwB$\text{R}_{388}$ and TMD$\text{T}_{\Delta}$CD$\text{T}_{\text{TrwB}}$, even if in the later belongs to another T4CP such as TraJ$\Delta$MK101. Regarding bands associated to $\beta$-sheet and $\beta$-turns, it is remarkable the absence of a band around 1661–1665 cm$^{-1}$ in TMD$\text{T}_{\Delta}$CD$\text{T}_{\text{TrwB}}$, as it was observed in TrwB$\text{R}_{388}$, TrwB$\Delta$N50, and TrwB$\Delta$N70. Nevertheless, the total proportion of the different bands associated to $\beta$-sheet and $\beta$-turns of TMD$\text{T}_{\Delta}$CD$\text{T}_{\text{TrwB}}$ (31%) is similar to the proportion seen in the soluble mutant proteins and significantly lower than that of TrwB$\text{R}_{388}$ (59%). Finally, a sizeable proportion (35%) of the structure of TMD$\text{T}_{\Delta}$CD$\text{T}_{\text{TrwB}}$ gave off a signal centered at 1640 cm$^{-1}$ (assigned to flexible, non-periodic elements) as seen in the deletion mutant proteins TrwB$\Delta$N50 and TrwB$\Delta$N70 but not in TrwB$\text{R}_{388}$ (Vecino et al., 2012). Previous studies about TrwB$\Delta$N70 and TrwB$\Delta$N50 showed that this band at 1640 cm$^{-1}$ also had a $\beta$-sheet component (Vecino et al., 2012). And it was published that at higher temperatures the band at 1640 cm$^{-1}$ split showing a $\beta$-sheet related band, which would not happen if the band was purely composed of unordered structures (Andraka et al., 2017). To elucidate if this also happened in TMD$\text{T}_{\Delta}$CD$\text{T}_{\text{TrwB}}$, IRS experiments at different temperatures were performed (20, 40, 60, and 80°C). With the increase of temperature, the 1640 cm$^{-1}$ band shifts to 1645 cm$^{-1}$ and that at 1626 cm$^{-1}$ increases both its contribution and width, suffering also a shift to higher wavenumbers. This behavior indicates that although those two not resolved bands included in that at 1640 cm$^{-1}$ do not directly split into two bands, there is a transfer of the $\beta$-sheet contribution to the 1626 cm$^{-1}$ only $\beta$-sheet band, confirming that the 1640 cm$^{-1}$ band in the chimeric protein was composed of both unordered and $\beta$-sheet elements.

With the aim of further studying if the deletion of the TMD has the same effect in all T4CPs, the same study was carried out with MobB$\text{C}_{\text{lofD13}}$ and its deletion mutant MobB$\Delta$TMD to be compared with TrwB$\text{R}_{388}$ and its variants. Figure 2A shows the original spectra and their curve-fitting decomposition corresponding to MobB$\text{C}_{\text{lofD13}}$ purified in the presence of detergent and its deletion mutant MobB$\Delta$TMD. In this case, both proteins exhibited four main bands. Band position, percentage area, and structure assignment corresponding to the deconvolved spectrum of the amide I region are summarized in Table 5. It can be observed that the component at 1653 and 1652 cm$^{-1}$ was assigned to $\alpha$-helix in MobB$\text{C}_{\text{lofD13}}$ and MobB$\Delta$TMD, respectively. But it should be pointed out that the proportion of the $\alpha$-helix in MobB$\Delta$TMD (29%) was lower than in native protein (35%), as expected taking into account the three transmembrane $\alpha$-helices postulated for the native protein (Figure 1A). The bands at 1666 or 1665 cm$^{-1}$ were associated with $\beta$-turns which were also observed in the proteins studied in Vecino et al. (2012), but not in TMD$\text{T}_{\Delta}$CD$\text{T}_{\text{TrwB}}$. Nevertheless, proportions were slightly different (13 and 22% for MobB$\text{C}_{\text{lofD13}}$ and MobB$\Delta$TMD, respectively). On the contrary, the signal centered at 1677 or 1682 cm$^{-1}$ assigned to low-frequency vibrations of $\beta$-sheet and $\beta$-turns showed similar proportions in MobB$\text{C}_{\text{lofD13}}$, MobB$\Delta$TMD (7 and 3%, respectively) and in the proteins studied in Vecino et al. (2012) but not in TMD$\text{T}_{\Delta}$CD$\text{T}_{\text{TrwB}}$ where the proportion of this component was 14%. Finally, as observed in TMD$\text{T}_{\Delta}$CD$\text{T}_{\text{TrwB}}$, TrwB$\Delta$N50 and TrwB$\Delta$N70, both MobB$\text{C}_{\text{lofD13}}$ and MobB$\Delta$TMD had

### Table 3: Conjugation and dominance experiments with TMD$\text{T}_{\Delta}$CD$\text{T}_{\text{TrwB}}$

| Plasmids in donors | T4CP | Transfer frequency |
|--------------------|------|--------------------|
| R388 (+) Wild type TrwB$\text{R}_{388}$ | 2.1 10$^{-1}$ |
| R388$\Delta$trwB (pSU1456) (–) | Ø | < 10$^{-8}$ |
| pKM101 (+) Wild type TraJ$\Delta$MK101 | 2.2 10$^{-1}$ |
| pKM101$\Delta$traJ (–) | Ø | < 10$^{-8}$ |
| R388$\Delta$trwB (pSU1456) TMD$\text{T}_{\Delta}$CD$\text{T}_{\text{TrwB}}$ | 1.82 10$^{-4}$ |
| pUB04 | TMD$\text{T}_{\Delta}$CD$\text{T}_{\text{TrwB}}$ | < 10$^{-8}$ |
| pUB04 (K142T) | TMD$\text{T}_{\Delta}$CD$\text{T}_{\text{TrwB}}$ | < 10$^{-8}$ |
| R388 pUB04 Wild type TrwB$\text{R}_{388}$ TMD$\text{T}_{\Delta}$CD$\text{T}_{\text{TrwB}}$ | 1.13 10$^{-2}$ |
| pKM101 pUB04 Wild type TraJ$\Delta$MK101 TMD$\text{T}_{\Delta}$CD$\text{T}_{\text{TrwB}}$ | 1.10 10$^{-2}$ |

Additionally, the effect of the chimeric protein in the conjugative process of R388 and pKM101 has been analyzed (dominance assays, shaded in gray). E. coli DH5$\alpha$ and UB1637 strains were used as donor and recipient cells, respectively. Transfer frequencies were normalized to the number of transconjugants per donor and are the mean value of at least five independent experiments. (+) Positive control; (–) Negative control; Ø: no T4CP.

### Table 4: Mobilization experiments.

| Plasmids in donors | T4CP | Transfer frequency |
|--------------------|------|--------------------|
| R388$\Delta$trwB (pSU1456) | Wild type MobB$\text{C}_{\text{lofD13}}$ | 2.42 10$^{-2}$ |
| pSL4833 (+) | MobB$\text{C}_{\text{lofD13}}$ | < 10$^{-8}$ |
| R388$\Delta$trwB (pSU1456) | MobB$\text{C}_{\text{lofD13}}$ | 1.75 10$^{-2}$ |
| pOPINE-mobB | MobB$\text{C}_{\text{lofD13}}$ | < 10$^{-8}$ |
| R388$\Delta$trwB (pSU1456) | Ø | < 10$^{-8}$ |

Transfer frequency of pSU4833 plasmid complemented with MobB$\text{C}_{\text{lofD13}}$ or MobB$\Delta$TMD mediated by the secretion channel of R388 conjugative system has been studied. E. coli DH5$\alpha$ and UB1637 strains were used as donor and recipient cells, respectively. Transfer frequencies were normalized to the number of transconjugants per donor and are the mean value of at least five independent experiments. (+) Positive control; (–) Negative control; Ø: no T4CP.
FIGURE 2 | (A) Amide I region of the infrared spectra of TMD\textsubscript{TraJ CD\textsubscript{TrwB}}, MobB\textsubscript{CloDF13}, and MobB\textsubscript{ATMD}. Proteins were purified, dialyzed against the corresponding buffer in D\textsubscript{2}O and analyzed by IR spectroscopy as explained in “Materials and Methods” section. Obtained spectra were curve-fitted to show the different secondary structure components as detailed in Table 5. (B) Thermal denaturation of TMD\textsubscript{TraJ CD\textsubscript{TrwB}}, MobB\textsubscript{CloDF13}, and MobB\textsubscript{ATMD} as seen by IR spectroscopy. The widths at half-height (WHH) of the amide I bands are plotted as a function of temperature (°C). Thermal denaturation is marked by an abrupt increase in bandwidth. Mid-point denaturation temperature (Tm) values corresponding to each protein are detailed in Table 5.

TABLE 5 | Secondary structure components and mid-point denaturation temperatures (Tm) of TrwB\textsubscript{R388}, TMD\textsubscript{TraJ CD\textsubscript{TrwB}}, TMD\textsubscript{ATMD}, TMD\textsubscript{TraJ CD\textsubscript{TrwB}}, MobB\textsubscript{CloDF13}, and MobB\textsubscript{ATMD}.

| Assignment | TrwB\textsubscript{R388} \textsuperscript{a} | TMD\textsubscript{TraJ CD\textsubscript{TrwB}} | TrwB\textsubscript{AN50} \textsuperscript{a} | TrwB\textsubscript{AN70} \textsuperscript{a} | MobB\textsubscript{CloDF13} | MobB\textsubscript{ATMD} |
|------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| \(\beta\)-T + \(\beta\)-S | 1676 5 | 1671 14 | 1676 2 | 1675 8 | 1677 7 | 1682 3 |
| \(\beta\)-T | 1661 27 | / | 1665 11 | 1665 11 | 1666 13 | 1665 22 |
| \(\alpha\)-H | 1647 41 | 1654 35 | 1654 27 | 1653 26 | 1653 35 | 1652 29 |
| Unordered + \(\beta\)-S | / | 1640 30 | 1641 37 | 1640 39 | 1639 29 | 1639 30 |
| \(\beta\)-S | 1631 27 | 1626 17 | 1628 17 | 1627 12 | / | / | / |
| Tm (°C) | 48 51 | 48 63\textsuperscript{b} | 56 62 |

Secondary structure components were obtained from band decomposition of the amide I band of IR spectra in D\textsubscript{2}O medium. The spectra are shown in Figure 2. Only areas above 5% are shown. For details on assignments, see the text. \textsuperscript{a}Data from Vecino et al. (2011, 2012). \textsuperscript{b}Unpublished data from our group. \(\beta\)-T, \(\beta\)-turns; \(\beta\)-S, \(\beta\)-sheet; \(\alpha\)-H, \(\alpha\)-helix; Tm, mid-point denaturation temperature.

A band at 1639 cm\textsuperscript{-1} assigned to unordered structures that represented 30% of the structure, a band that is missing in TrwB\textsubscript{R388}.

Thermal Stability of T4CPs and Their Variants

The information regarding the denaturation of TMD\textsubscript{TraJ CD\textsubscript{TrwB}}, MobB\textsubscript{CloDF13}, and MobB\textsubscript{ATMD} was obtained through analysis of the amide I band (Figure 2B). Specifically, two bands appear at 1615–1620 cm\textsuperscript{-1} and 1680 cm\textsuperscript{-1} when the protein aggregates. The appearance of these bands allows monitoring the denaturation process of the protein and the calculation of the mid-point denaturation temperature (Tm) (Table 5). Data treatment and band decomposition of the original amide I have been described previously (Vecino et al., 2011, 2012).

The thermal stability of TMD\textsubscript{TraJ CD\textsubscript{TrwB}} purified in detergent was compared to TrwB\textsubscript{R388}, TrwB\textsubscript{AN50}, and TrwB\textsubscript{AN70} (Vecino et al., 2012). As observed in Figure 2B, the thermal denaturation of TMD\textsubscript{TraJ CD\textsubscript{TrwB}} started at 35°C showing a mid-point denaturation temperature of 51.1°C at the tested conditions. This result is similar to TrwB\textsubscript{R388} and to the TrwB\textsubscript{AN50} (Vecino et al., 2012; Table 5).

Thermal stability of MobB\textsubscript{CloDF13} and MobB\textsubscript{ATMD} was also studied by IR spectroscopy. As depicted in Figure 2B, the denaturation of the native protein starts at 47°C, achieving its Tm...
at 56°C, while the denaturation of MobBΔTMD starts at 55°C, achieving its Tm at 62°C (Table 5).

**Subcellular Location**

Studies about subcellular location of different T4CPs reported up to now have rendered ambiguous results (Kumar and Das, 2002; Gunton et al., 2005; Segura et al., 2014). To gain a deeper knowledge of this matter, specifically regarding the role of the TMD, we have studied the subcellular location of TMD\_TraJ\_CD\_TrwB, MobB\_CloDF13, and MobB\_ΔTMD under different experimental conditions. Subcellular location was analyzed by confocal fluorescence microscopy using eGFP-labeling and immunofluorescence techniques. Since eGFP proteins are only fluorescent when they are properly folded, their visualization ensures the analysis of functional proteins, excluding those that are denatured or included in inclusion bodies (Drew et al., 2006). In both studies similar results were obtained but eGFP-labeling rendered better quality images (Supplementary Figure S2).

To study the effect of expression times in the absence of the rest of T4SS proteins, the subcellular location of each protein was visualized after induction with 1 mM IPTG for 4 or 20 h. Different patterns were observed in the location of each protein at different expression times (Figure 3 and Table 6). After 4 h of expression, TrwB\_R388 and TMD\_TraJ\_CD\_TrwB were mainly located along the whole cell membrane and switched to a single-pole after 20 h, being this change less pronounced in the case of the chimeric protein. MobB\_CloDF13 was predominantly located at both poles both at 4 and 20 h, showing a little increase in one pole location after 20 h (Figure 4 and Table 6). On the contrary, most of the cells (95%) showed MobB\_ΔTMD located on a single pole in the cytosol at both tested times.

Since the interaction with other conjugative proteins of the T4SS could modify the subcellular location pattern (Segura et al., 2014), the proteins that were active in vivo (i.e., TrwB\_R388, TMD\_TraJ\_CD\_TrwB, and MobB\_CloDF13) were also observed in the presence of a T4SS lacking a functional T4CP that could interfere with the studied eGFP variant. On the one hand, TrwB\_R388 and TMD\_TraJ\_CD\_TrwB were analyzed in the presence of the remaining conjugative proteins of R388 (i.e., in the presence of plasmid pSU1456 that缺乏 functional TrwB\_R388 but contains the remaining conjugative proteins). On the other hand, since CloDF13 needs the T4SS of a co-resident conjugative plasmid to be mobilized, the subcellular location of MobB\_CloDF13 was studied in the presence of R388 lacking functional TrwB\_R388 (plasmid pSU1456) and also in the presence of both, R388 lacking functional TrwB\_R388 (plasmid pSU1456) and the mobility region of CloDF13 lacking functional MobB\_CloDF13 (plasmid pSU4833) (Table 6). In these experiments (Figures 3, 4) the location pattern of each protein was the same in the absence or presence of a conjugative system (Table 6). However, although MobB\_CloDF13 kept two poles as its main location in all the tested conditions, in the presence of R388 lacking functional TrwB\_R388 it did not partially switch to a single-pole after 20 h, as in the absence of it.

The general observed pattern for all studied T4CPs was that the presence of T4SS enhanced the percentage of cells with the T4CP at the predominant location shown in the absence of the T4SS for each protein after 20 h (Figures 3, 4). Specifically, the percentage of cells showing TrwB\_R388 and TMD\_TraJ\_CD\_TrwB at a single pole increased and so did the percentage of cells showing MobB\_CloDF13 at both poles. Moreover, regarding MobB\_CloDF13, the additional presence of its cognate mobilization region enhanced the effect produced by T4SS\_R388.


**TABLE 6 | Subcellular location of different T4CP-eGFP fusion-proteins at different expression times in the absence or presence of T4SSR388.**

| Expression time (h) | Without T4SS (%) | T4SSR388 (%) | T4SSR388 and MOBCloDF13 (%) |
|---------------------|------------------|--------------|-----------------------------|
|                      | M 1P 2P          | M 1P 2P      | M 1P 2P                     |
| TrwBR388            | 97 (77) 11 (9) 18 (14) | 34 (84) 3 (8) 3 (8) | – |
| 20                  | 31 (15) 155 (75) 29 (10) | 10 (6) 138 (90) 6 (4) | – |
| TMDtrajCDtrwB       | 85 (83) 11 (11) 6 (6) | 55 (76) 13 (18) 4 (6) | – |
| 20                  | 49 (40) 62 (51) 10 (6) | 18 (14) 85 (67) 23 (18) | – |
| MobBCloDF13         | 18 (16) 8 (8) 73 (74) | 22 (19) 16 (14) 80 (68) | 7 (5) 19 (13) 122 (82) |
| 20                  | 20 (18) 24 (21) 69 (61) | 16 (15) 16 (15) 74 (70) | 7 (4) 43 (22) 143 (74) |
| MobBΔTMDa           | 0 40 (95) 2 (5) | N.d. | N.d. |
| 20                  | 0 35 (95) 2 (5) | N.d. | N.d. |

The eGFP variants of the proteins in the first column were expressed in E. coli BL21 (DE3) strain by induction with 1 mM IPTG for 4 and 20 h. The numbers represent the amount of cells showing each location. Total number of cells counted for each sample was between 40 and 200. Numbers between parentheses represent the percentages calculated up to the total amount of cells counted for each sample. The main location for each condition is marked in bold. M, location in the periphery of the cell membrane; 1P, location at a single-pole; 2P, location at both poles. –, without relevance for this study. N.d., not determined. aPolar location in the cytosol.

**DISCUSSION**

The increase of multidrug-resistant bacteria has become one of the major health concerns in our society (World Health Organization [WHO], 2019), being bacterial conjugation one of the key mechanisms responsible for the spread of antibiotic resistance genes among bacteria (Bello-López et al., 2019). This process is performed through a T4SS, a multiprotein complex that transfers the nucleoprotein substrate from a donor into a recipient bacterium (Waksman, 2019). T4CPs are essential proteins during conjugation, as they connect the substrates to be transferred in the cytosol with the secretion machinery in the membrane (Gomis-Ruth et al., 2005). Despite their importance, as membrane proteins are challenging to be studied, their characterization has been mostly accomplished using mutants that lack their TMD (Schröder and Lanka, 2003; Tato et al., 2007; Larrea et al., 2017). However, several studies performed with TrwBR388, the full-length T4CP of the conjugative plasmid R388, have proven that the TMD is more than a mere anchor to the membrane and that it has a role in protein activity, stability, oligomerization and subcellular localization (Moncallán et al., 1999; Horrache et al., 2002, 2004, 2006; Vecino et al., 2010, 2011; Segura et al., 2013, 2014).

The aim of this work has been to provide new data about different T4CPs that will contribute to infer general conclusions on their functioning to develop strategies to inhibit them and control the spread of antibiotic resistance genes. To do so, we studied the in vivo functionality, secondary structure, thermal stability, and subcellular location of TrwBR388, its chimeric protein TMDtrajCDtrwB, and the T4CP of the mobilizable plasmid CloDF13, MobBCloDF13, and its TMD-less mutant, MobBΔTMD.

**In vivo Functionality**

TMDtrajCDtrwB was able to complement the ΔtrwB mutation for R388 transfer, although with a lower transfer frequency than TrwBR388. This decrease in conjugation frequency may be due to a combination of effects such as conformational changes in the CD of TrwBR388 due to its chimeric nature that render a less active protein and the heterologous interaction between TMDtraj and the T4SS of R388 (Llosa et al., 2003). Thus, TMDtrajCDtrwB-mediated transfer of R388 would occur through a specific interaction between CDtrwB with its cognate relaxase, TrwC388, and an unspecific interaction of TMDtraj with the heterologous T4SS from R388. This could explain why TMDtrajCDtrwB did not complement the ΔtraI mutation (pKM101ΔtraI) since CDtrwB could not recognize the heterologous TraIPKM101 relaxase, even if TMDtraj interacted with its cognate T4SSpKM101.

Moreover, TMDtrajCDtrwB showed negative dominance in the presence of the native T4CPs TrwBR388 or TraIPKM101. Since it has been reported that TMDtraj can interact with both T4SSR388 and T4SSpKM101 (Llosa et al., 2003; De Paz et al., 2010; Celaya et al., 2017), a possible explanation for the observed negative dominance could be that competition for the T4SS occurred. According to this hypothesis, TMDtrajCDtrwB would have interacted with the secretion channel, queuing it from interacting with the native T4CPs and reducing the conjugative rate of the wild type system. This hypothesis comes into agreement with the fact that the transfer of pKM101 using TraIPKM101 and T4SSR388 is one order of magnitude lower than using T4SSpKM101 (Llosa et al., 2003). Another explanation compatible with dominance experiments with both systems would be that non-functional heteroligomers were made between the native and the chimera proteins, competing for the conjugative machinery and therefore lowering the transfer frequency. Any of these alternatives or a combination of them would have caused a decrease in the plasmid transfer rate, as observed in the dominance mating assays. It must be underlined that a point mutation in the Walker A domain, TMDtrajCDtrwB (K142T), resulted in a non-functional phenotype. Hence, the K mutation totally arrested transfer capacity as previously reported for other T4CPs (Moncallán et al., 1999; Kumar and Das, 2002; Gunton et al., 2005), suggesting that concerning its NBD TMDtrajCDtrwB is functionally similar to TrwBR388 despite the results obtained in the complementation and dominance studies.
Regarding CloDF13 system, it was observed that the deletion of the TMD rendered a non-functional MobBΔTMD as it occurs with other TMD-less mutants such as TrwBΔN70 and PcfCΔN103 (Chen et al., 2008). However, a TcpA mutant lacking the TMD, but not the N-term cytosolic residues, TcpA166-104, was able to perform conjugation, although at a frequency lower than the wild type plasmid. In this regard, unpublished experiments with TrwBΔN8, which lacks the N-term cytosolic eight residues, showed a decrease in the transfer rate of more than three orders of magnitude (Vecino, 2009). Taken together these results, it seems that not only the TMD but also the N-term cytosolic residues have an important role in the transfer capacity of T4CPs. In this context, the importance of this small region in specific interactions with the relaxosome has already been described (Llosa et al., 2003; Schroder and Lanka, 2003). In the case of MobBCloDF13 its N-term is located in the periplasm (Figure 1A), which could be an important feature for recognition and interaction with conjugative secretion channels.

**Secondary Structure and Thermal Stability**

As it has been described that the TMD influences the secondary structure of the CD and the thermal stability of TrwB1388 (Hormaeche et al., 2004; Vecino et al., 2011, 2012), in this work we have studied whether this behavior can be observed in TMD\_Traj\_CD\_TrwB and in MobBCloDF13.

Regarding TMD\_Traj\_CD\_TrwB, one of the most important differences in comparison with TrwB1388 was the appearance of a band at 1640 cm\(^{-1}\), mainly assigned to flexible structures (non-periodic elements) related to a less compact overall structure (Eichabe et al., 1998; Agopian et al., 2016). As the crystal structure of TrwBΔN70 shows flexible loops (Gomis-Rüth et al., 2002), the presence of the 1640 cm\(^{-1}\) band present at similar percentages (30-37\%) in TMD\_Traj\_CD\_TrwB\_R388, TrwBΔN50, and TrwBΔN70 mutant proteins but missing in TrwB1388, could be explained as the loss of the compact structure of TrwB1388 due to the deletion of its cognate TMD (Hormaeche et al., 2004).

Taking into account the values related to all the bands associated to β-sheet elements (1671, 1640, and 1626 cm\(^{-1}\)) it could be concluded that the total percentage of β-sheets in TMD\_Traj\_CD\_TrwB is just slightly smaller to that of TrwB1388. Concerning β-turns, the band assigned to them in the TrwB1388 related proteins, 1661-1665 cm\(^{-1}\), was not observed in TMD\_Traj\_CD\_TrwB. However, it can be postulated that the increase seen in TMD\_Traj\_CD\_TrwB of the band at 1671 cm\(^{-1}\), was partially related to the β-turns component seen in the mutants at 1665 cm\(^{-1}\). These would imply that the β-turns component of the chimeric protein is lower than the one of the native protein and similar to the TMD deletion mutants. Finally, TMD\_Traj\_CD\_TrwB shows a decrease in α-helix percentage (35%) in comparison with TrwB1388 (41%), but an increase in comparison to the mutants (26%).

Therefore it seems that TMD\_Traj\_CD\_TrwB presents qualitative and quantitative features in between the native protein and the deletion mutants. These results suggest that the presence of a heterologous full-length TMD does provide a more compact and ordered structure to the T4CP in comparison to the TMD-less mutants, even if it does not reach the level of the native...
protein. This result comes in agreement with the transfer capacity reduction of the chimeric protein that could be explained partially by the observed structural changes reported here.

To test if the results described for TrwB<sub>R388</sub> could be extrapolated to other T4CPs, the secondary structures of MobB<sub>CloDF13</sub> and MobBΔTMD were studied (Table 5). Surprisingly, MobBΔTMD presented an IR spectrum similar to that obtained for MobB<sub>CloDF13</sub>. It presented smaller helical structure percentages and higher β-turns percentages but both showed similar unordered and β-sheet percentages. This suggests that in MobB<sub>CloDF13</sub> the presence of the TMD does not have an effect on the structure of the CD as it does in TrwB<sub>R388</sub>. As MobB<sub>CloDF13</sub> has to interact with heterologous T4SSs, it could be that its TMD has to interact with heterologous T4SSs without altering the structure of its CD where the specific interaction with its cognate relaxosome occurs.

Concerning the thermal denaturation of TMD<sub>TraJ</sub>CD<sub>TwbB</sub>, its Tm was similar to the ones described for TrwB<sub>R388</sub> and the mutant lacking the first transmembrane helix, TrwBΔN50 (Table 5). This result suggests that although the secondary structures of the mutants differ from that of the native protein, their overall thermal stability is similar. Additionally, when comparing the results between both studied systems, as expected due to the high instability of purified membrane proteins (González Flecha, 2017) the soluble proteins showed higher mid-point denaturation temperatures than the full-length proteins. Specifically, MobBΔTMD and TrwBΔN70 showed similar Tm values (Tm 62 and 63°C, respectively); on the contrary, MobB<sub>CloDF13</sub> was more stable than TrwB<sub>R388</sub> against thermal denaturation (Tm 56 and 48°C, respectively). This could be related to different buffer compositions that had to be used when MobB<sub>CloDF13</sub> and TrwB<sub>R388</sub> were analyzed.

### Subcellular Location

The polar location of proteins in bacteria underlines their sophisticated internal organization, being important in many processes like chemotaxis and cellular division (Howard, 2004). Similarly, subcellular location has been considered important in bacterial conjugation (Chen et al., 2008; Leonetti et al., 2015). Sequence analysis, cell fractionation, and protein purification experiments proved that TrwB<sub>R388</sub>, TMD<sub>TraJ</sub>CD<sub>TwbB</sub>, and MobB<sub>CloDF13</sub> are located in the bacterial membrane, while MobBΔTMD is located in the cytosol (data not shown). As studies in the literature do not show a consensus pattern either in the location of the T4CPs nor in the role of each domain in this property (Kumar and Das, 2002; Bauer et al., 2011; Segura et al., 2014), the subcellular location of TrwB<sub>R388</sub>, TMD<sub>TraJ</sub>CD<sub>TwbB</sub>, MobB<sub>CloDF13</sub>, and MobBΔTMD was investigated.

In this work we observed TrwB<sub>R388</sub> located along the membrane after 4 h of induction and only after 20 h it focused at single pole. These results differ from our previous results where the polar location of TrwB<sub>R388</sub> was observed after 4 h (Segura et al., 2014). However, the induction OD<sub>600</sub> values were different (0.4 vs. 0.7, this work and previous work, respectively), probably rendering populations at different growth phase. On the contrary MobB<sub>CloDF13</sub> was located at both poles in the membrane at 4 and 20 h after induction.

Previous studies have reported that in the absence of other conjugative proteins, T4CPs that lacked the whole TMD or even the periplasmic loop located in the cytosol or at the membrane periphery, respectively (Kumar and Das, 2002; Segura et al., 2014). Moreover, the TMD alone of TrwB<sub>R388</sub> located at the membrane poles without the need for the CD (Segura et al., 2014), suggesting a leading role for the TMD in the subcellular location of the T4CPs. Surprisingly, MobBΔTMD located at a single cell pole in the cytosol even in the absence of other conjugative proteins. These results suggest that mobilizable plasmid-related T4CPs could use different mechanisms than VirD4-type T4CPs for subcellular location. It has been speculated that the polar location of T4CPs could be related to interactions with the cardiolipin enriched membrane poles (Mileykovskaya and Dowhan, 2009; Segura et al., 2014), but at the same time mediated by complex and dynamic changes in transduction, cytoskeleton proteins, etc. (Shapiro et al., 2002). Since interactions between mobilizable plasmid-related T4CPs and T4SSs are not specific, these T4CPs could have evolved to develop different mechanisms to interact with the membrane and ensure their polar location.

Previous studies (Kumar and Das, 2002; Gunton et al., 2005; Segura et al., 2014) have reported that the location of native T4CPs is independent of the presence of the rest of the conjugative proteins. Our results suggest that the presence of a complete conjugative system (i.e., mobilization region and secretion channel) seems to enhance the polar location of wild type T4CPs. Moreover, although the location of MobB<sub>CloDF13</sub> at both poles was enhanced in the presence of T4SS<sub>S</sub><sub>388</sub>, it was further enhanced when MOB<sub>CloDF13</sub> was also present.

Taking all together, it seems that that no universal location patterns can be attributed to T4CPs. Nevertheless, three conclusions can be undertaken regarding subcellular location: (i) T4CPs localize either at a single pole or both poles, depending on the system; (ii) the presence of a TMD is not essential for the polar location of a mobilizable plasmid associated T4CP, and (iii) the presence of a conjugative system enhances the polar location of full-length T4CPs.

To sum up, the comparative study between the conjugative system related TrwB<sub>R388</sub> and the mobilizable plasmid-related MobB<sub>CloDF13</sub> and their variants has underlined that the characteristics described for the paradigmatic conjugative plasmid related VirD4-type T4CPs and their TMDs should not be ascribed to the whole T4CP family.

### DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation, to any qualified researcher.

### AUTHOR CONTRIBUTIONS

IÁ-R, CG, and IAl contributed to the design of the work (text and figures) and the acquisition of the data, writing and revision of the content, approval of the
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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmolb.2020.00185/full#supplementary-material
Howard, M. (2004). A mechanism for polar protein localization in bacteria. *J. Mol. Biol.* 335, 655–663. doi: 10.1016/j.jmb.2003.10.058

Kumar, R. B., and Das, A. (2002). Polar location and functional domains of the *Agrobacterium tumefaciens* DNA transfer protein VirD4. *Mol. Microbiol.* 43, 1523–1532. doi: 10.1046/j.1365-2958.2002.02829.x

Langer, P. J., and Walker, G. C. (1981). Restriction endonuclease cleavage map of DNA transfer protein VirD4. *Mol. Microbiol.* 335, 655–663. doi: 10.1016/j.jmb.2006.04.059

Lee, C. A., et al. (2015). Critical components of the conjugation machinery of the integrative and conjugative element ICEBs1 of *Bacillus subtilis*. *Proc. Natl. Acad. Sci. U.S.A.* 112, 13928–13933. doi: 10.1073/pnas.1515692112

Moncalián, G., Cabezón, E., Alkorta, I., Valle, M., Moro, F., Valpuesta, J. M., Nuñez, B., and de la Cruz, F. (2001). Two atypical mobilization proteins are coupling proteins in type IV secretion. *EMBO J.* 20, 9662–9669. doi: 10.1093/emboj/20.24.9662

Vecino, A. J., Segura, R. L., Ugarte-Uribe, B., Vecino, A. J., de la Cruz, F., Goñi, F., et al. (2004). Subcellular location of the coupling protein TrwB and the role of its translational-domain. *Biochim. Biophys. Acta Biomembr.* 1788, 2160–2169. doi: 10.1016/j.bbamem.2007.07.005

Waksman, G. (2019). From conjugation to T4S systems in Gram-negative bacteria: a mechanistic biology perspective. *EMBO Rep.* 20:e47012.

Wald, G. S., Standish, B. M., Berendzen, J., and Twerdill, T. C. (1999). Rapid protein-chemical assay using green fluorescent protein. *Nat. Biotechnol.* 17, 691–695. doi: 10.1038/10904

Whitaker, N., Berry, T. M., Rosenthal, N., Gordon, J. E., Gonzalez-Rivera, C., Sheehan, K. B., et al. (2016). Chimeric coupling proteins mediate transfer of heterologous type IV effectors through the *Escherichia coli* pKM101-encoded conjugation machinery. *J. Bacteriol.* 198, 2701–2718. doi: 10.1128/jb.00378-16

World Health Organization [WHO] (2019). Thirteenth General Programme of Work 2019–2023. Geneva: World Health Organization [WHO].

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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