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Subcellular location of the coupling protein TrwB and the role of its transmembrane domain

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ABSTRACT

Conjugation is the most important mechanism for horizontal gene transfer and it is the main responsible for the successful adaptation of bacteria to the environment. Conjugative plasmids are the DNA molecules transferred and a multiprotein system encoded by the conjugative plasmid itself is necessary. The high number of proteins involved in the process suggests that they should have a defined location in the cell and therefore, they should be recruited to that specific point. One of these proteins is the coupling protein that plays an essential role in bacterial conjugation. TrwB is the coupling protein of R388 plasmid that is divided in two domains: i) The N-terminal domain referred as transmembrane domain and ii) a large cytosolic domain that contains a nucleotide-binding motif similar to other ATPases. To investigate the role of these domains in the subcellular location of TrwB, we constructed two mutant proteins that comprised the transmembrane (TrwBTM) or the cytoplasmic (TrwBΔN70) domain of TrwB. By immunofluorescence and GFP-fusion proteins we demonstrate that TrwB and TrwBTM mutant protein were localized to the cell pole independently of the remaining R388 proteins. On the contrary, a soluble mutant protein (TrwBΔN70) was localized to the cytoplasm in the absence of R388 proteins. However, in the presence of other R388-encoded proteins, TrwBΔN70 localizes uniformly to the cell membrane, suggesting that interactions between the cytosolic domain of TrwB and other membrane proteins of R388 plasmid may happen. Our results suggest that the transmembrane domain of TrwB leads the protein to the cell pole.

1. Introduction

Bacterial conjugation is a contact-dependent process during which a DNA molecule (usually a conjugative plasmid) is transferred from a donor to a recipient bacterium. Conjugation greatly increases prokaryotic genome plasticity by transmitting important traits for survival in adverse environments. In particular, conjugation is of great importance in human health because antibiotic resistance genes are the most common genes transmitted through this mechanism among pathogenic bacteria [1–3].

Conjugative plasmids encode the proteins necessary for DNA processing, recruitment, and translocation [1]. DNA processing is initiated at the origin-of-transfer (*oriT*) sequence where the relaxase introduces a nick in the T-DNA and remains covalently bound at the 5′ end of DNA to render a DNA–protein complex called relaxosome [4–6]. Relaxase also guides the ssDNA to the coupling protein (T4CP), which is thought to use its ATP hydrolysis activity to pump DNA across the type IV secretion system [7]. Type IV secretion systems (T4SS) are large protein complexes that transverse the cell envelope of many bacteria [8]. Recently published studies provide important structural features of these complexes and about their assembly [9–12]. The large size and the large number of proteins involved in the assembly suggests that these systems have a defined location in the cell and should be recruited to that specific point. Subcellular location studies in different systems indicate that T4SS proteins localize to the cell membrane, sometimes distributed along the cell membrane or localized to a few sites on the bacterial membrane or in polar foci to the cell pole [13–18].

As previously stated, T4CPs are responsible for bridging the relaxosome (DNA–protein complex) with the secretion system (T4SS) [19,20] and they are essential for bacterial conjugation. They are polytopic inner-membrane proteins with two transmembrane helices [21–23]. In particular, TrwB is the coupling protein of the *E. coli* R388 conjugative plasmid and up to date it is the most widely studied member of the family. Structural [24–26], biochemical [27–32], and biophysical [33–35]

Abbreviations: T4SS, type IV secretion system; T4CP, type IV coupling protein; TMD, transmembrane domain; NBD, nucleotide-binding domain; IFM, immunofluorescence microscopy

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studies have shed light on its biological activity and on the role of the transmembrane domain of TrwB in its biological function. Cellular location of coupling proteins could be related to their role as connectors of the DNA-substrate and the transfer apparatus but up to day, only the subcellular locations of TraG of R27 [16] and VirD4 of *Agrobacterium tumefaciens* [14] have been reported. Nevertheless there is a discrepancy between both studies and while TraG is located at discrete foci in the cell periphery independently of R27 conjugative proteins [16], VirD4 localizes to the cell poles without the other essential conjugation proteins [14].

In this study, subcellular location of TrwB was investigated by immunofluorescence microscopy (IFM) and GFP-fusion protein labelling microscopy. To study the role of its different domains (*i.e.*, cytosolic and transmembrane domains) two deletion mutants were investigated: the soluble mutant protein TrwBΔN70 that contains the cytosolic domain of TrwB and the TrwBTM mutant protein that contains the transmembrane domain of TrwB.

2. Materials and methods

2.1. Bacterial strains

For conjugation experiments, *E. coli* DH5α [36] was used as donor and *E. coli* UB1637 cells [37] were the recipients. *E. coli* BL21C41 (DE3) or BL21C43 (DE3) strains [38] were used for location studies.

2.2. Plasmids

Table 1 shows the plasmids used in this work. They were constructed by inserting a PCR fragment digested by the respective restriction enzymes in a cloning vector as explained in Table 2 and using standard DNA recombination technology [43]. Genes encoding TrwB and its mutant proteins, TrwBΔN70 (amino acids 70–507) and TrwBTM (amino acids 1–77), were cloned in pET22b to add a His tag in the C-terminal of the protein. When GFP fusion proteins were constructed Waldo-e plasmid was used [40].

2.3. Conjugation assays

The mating protocol was similar to that previously reported [44] except for some modifications. Briefly, after a 1:20 dilution of an overnight culture, 500 μl of donors in different growth phases (*E. coli* DH5α cotransformed with the appropriate plasmid) and recipient cells (*E. coli* UB1637) were mixed, centrifuged and the pellet placed onto a GS Millipore filter (0.22-μm pore size) on a pre-warmed LB-agar plate for 1 h at 37 °C. After this, bacteria were washed from the filter, diluted in 2 ml LB, and suitable dilutions plated on selective media [plates containing streptomycin (50 μg/ml) and trimethoprim (10 μg/ml) for the transconjugants and only trimethoprim (10 μg/ml) for donors]. Conjugation frequencies were normalized for the number of transconjugants per donor cell.

2.4. Subcellular location of TrwB using GFP-fusion reporter proteins

TrwB-GFP (pUB9) and TrwBTM-GFP (pUB10) fusion proteins were constructed using the pWaldo-GFPe plasmid, where the GFP only fluoresces when the protein is correctly folded [40]. Cell cultures (10 ml LB supplemented with 0.1 mg/ml kanamycin) were grown at 37 °C with shaking. When A_{600} was 0.7, IPTG was added to a 1 mM concentration. Cells were incubated at 25 °C for 4, 6 or 20 h. Then 1 ml culture was centrifuged at 8000 ×*g* for 2 min, resuspended and fixed with 4% paraformaldehyde for 10 min. Samples were centrifuged at 8000 ×*g* for 5 min, the supernatant was removed and the pellet was resuspended in 1 ml PBS. Subsequently, 500 μl cells were diluted (1:20) in PBS and placed on a poly-L-lysine-coated coverslip that was then put into a 24-well plate and centrifuged for 10 min at 800 ×*g*. Next, the sample was incubated for 30 min at room temperature, the excess liquid was removed, and the coverslips were washed with PBS and mounted on glass slides. Finally, the slides were placed on top of an Olympus Fluoview FV500 inverted fluorescence confocal microscope, followed by image acquisition with a 60× oil immersion objective, using an excitation wavelength of 488 nm. The emission was recovered between 505–525 nm. Image treatment was performed using FV10-ASW 1.7 software.

2.5. Subcellular location of TrwB by immunofluorescence microscopy

The method described by Kumar and Das with slight modifications was used [14]. Briefly, cell cultures separately transformed with plasmids pUB3, pSU4637 or pUB7 were grown and induced at 25 °C with 1 mM IPTG for 1 h. Then 1 ml culture was centrifuged at 8000 ×*g* for 2 min, cells were resuspended and fixed with 4% paraformaldehyde for 10 min at room temperature. Samples were washed three times with PBS and the pellet was resuspended and incubated for 10 min at 4 °C with 1.5 ml 25 mM Tris–HCl (pH 7.8), 1.85% (w/v) glucose and 10 mM EDTA, supplemented with 2 mg/ml lysozyme. A 20 μl aliquot of the cell suspension was immediately spread on each poly-L-lysine-coated coverslip, incubated for 10 min and centrifuged at 3000 ×*g* for 5 min. Coverslips were then dipped in −20 °C methanol for 4 min and in −20 °C acetone for 20 s followed by extensive washing with PBS. A 200 μl aliquot of the blocking solution [2.5% (w/v) BSA in PBS] was added to each coverslip and incubated at 37 °C for 1 h. Coverslips with the fixed cells were rinsed 3 times in PBS and incubated overnight at 4 °C with 200 μl of rabbit anti-TrwB antiserum or mouse anti-His (C-term) monoclonal antibody (Invitrogen) as primary antibodies at a 1:200 dilution in PBS in both cases. Coverslips were washed 4 times in PBS before incubation in the dark with the appropriate fluorophore-conjugated secondary antibody in PBS for 3 h at room temperature. 10 μg/ml Alexa Fluor 488 goat anti-rabbit or Alexa

Table 1
Plasmids used in this work.

Plasmid	Description	Phenotype	Reference
pET22b(+)	Expression vector	Amp^R, C-Terminal^{-}His tag	Novagen
pET3a′	Expression vector	Amp^R, Rep (pMB8)	[39]
pSU4637	pET3a′::*trwBΔN70*	Amp^R, Rep (pMB8)	[24]
pUB3	pET22b(+)::*trwB*	Amp^R, His-tag	[29]
pWaldo-GFPe	Expression vector	Km^R, C-Terminal GFP and His tag	[40]
R388	Natural plasmid	Su^R, Tp^R, TRA_W, IncW	[41]
pSU1443	R388 except *trwB* gene	Su^R, Tp^R, TRA_W, IncW	[42]
pUB7	pET22b(+)::*trwBTM*	Amp^R, TrwBN77 C-Terminal^{-}His tag	This work
pUB9	pWaldo-GFPe::*trwB–GFP*	Km^R, TrwB C-Terminal GFP and His tag	This work
pUB10	pWaldo-GFPe::*trwBTM–GFP*	Km^R, TrwBTM C-Terminal GFP and His tag	This work

TRA_W, transfer region of the conjugative plasmid R388.
Amp^R, ampicillin resistance; Km^R, kanamycin resistance; Su^R, sulfonamide resistance; and Tp^R, trimethoprim resistance.

Table 2
Primers used in this work.

Plasmid name	Used for construct	Protein	Sequence (5′ → 3′)	Restriction enzyme used
pUB7	pET22b	TrwBTM	Forward: GGAATTCCATATGCATCCAGACGATCAA AGA	*Nde*I
			Reverse: CCGCCGCTCGAGTTCGCCTTGTCCGACGCT	*Xho*I
pUB9	pWaldo-GFPe	TrwB–GFP	Forward: CCGCCGCTCGAGATGCATCCAGACGAT CAAAGA	*Xho*I
			Reverse: CGCGGATCCGATAGTCCCCTCAACAAAGGCCGG	*Bam*HI
pUB10	pWaldo-GFPe	TrwBTM–GFP	Forward: CCGCCGCTCGAGATGCATCCAGACGAT CAAAGA	*Xho*I
			Reverse: CGCGGATCCTTCGCCTTGTCCGACGCTATT	*Bam*HI

Fluor 488 goat anti-mouse antibodies (both from Molecular Probes) were used as secondary antibodies.

3. Results

Among the different functions attributed to the T4CPs, one is the recruitment of the relaxosome to the T4SS [7,30,44,45]. Additionally, several studies of our group showed a role of the TrwB TMD in different biochemical and biophysical properties of TrwB (*i.e.*, nucleotide binding activity, hexamerization, stability, secondary structure elements) [27,29,32–35,46]. Therefore, the TMD could also play an important role in subcellular location of TrwB. Thus in this work we studied the subcellular location of TrwB, and of its deletion mutant proteins TrwBΔN70 and TrwBTM that contain the cytosolic or transmembrane domains, respectively.

3.1. Transfer capacity of GFP-fusion proteins

To determine the cellular location pattern of TrwB both immunolocalization [14] and GFP-fusion protein strategies were used. In this second approach Waldo-e plasmid was used. This plasmid is a folding reporter that will fluoresce only when GFP is correctly folded in the cytoplasm [40]. GFP-fusion approach has been broadly used to localize structural components of the conjugation machinery [15,47–50]. Nevertheless, the bulky nature of GFP can represent an inconvenience that could render non-functional fusion proteins. Because of that, before studying the location of TrwB and TrwBTM, we studied the transfer capacity of TrwB–GFP and TrwBTM–GFP fusion proteins. It was considered that if the fusion proteins showed similar transfer capacity than the corresponding non-fluorescent proteins they could be used in cellular location studies. As observed in Fig. 1, TrwB–GFP fully restored R388 transfer with a transfer frequency similar to the one presented by the wild-type protein (0.1 transconjugants/donor). As expected, TrwBTM–GFP, the mutant protein without nucleotide-binding domain (NBD), did not show any transfer capacity in agreement with the results obtained with the corresponding non-fluorescent mutant protein ($\leq 10^{-8}$ transconjugants/donor).

From these results we conclude that TrwB–GFP and TrwBTM–GFP fusion proteins can be used to examine their subcellular location.

3.2. TrwB localizes to the cell pole independently of R388 proteins

Subcellular location of TrwB was achieved using immunofluorescence microscopy. To visualize TrwB, cells were transformed with plasmid pUB3 containing *trwB* gene and treated as described in Materials and methods section. Rabbit anti-TrwB antiserum and Alexa Fluor 488 goat anti-rabbit antibodies were used as primary and secondary antibodies, respectively. It was observed that TrwB localized to the cell pole (arrowheads) in the absence and in the presence of the remaining R388-encoded conjugative proteins (Fig. 2, panels 1 and 2, respectively). Bacteria that did not contain *trwB* genes were used as negative controls in the absence and in the presence of the R388 plasmid (Fig. 2, panels 7 and 9 and panels 8 and 10, respectively). Additionally, cells treated in the same way except that primary and secondary antibodies were omitted were used as negative control (data not shown). In both control experiments no fluorescence was observed.

Similar results were observed when fusion protein TrwB–GFP was visualized by confocal microscopy. Bacteria expressing TrwB–GFP fusion protein exhibited foci at the cell pole (arrowheads). These polar foci were observed in the absence and in the presence of the remaining conjugative proteins of R388 encoded by pSU1443 plasmid (Fig. 3, panels 3 and 7, respectively). Quantitative analysis showed that 23% of the cells presented foci and 100% of these cells showed foci localized at a single pole (Table 3), indicating that TrwB localized to a single cell pole even when the remaining conjugative proteins of R388 were absent. The fluorescence was standardized and cells that did not express *trwB–GFP* gene did not show any fluorescence (Fig. 3, panels 1, and 5). It was also observed that GFP was solely found in the cytosol (data not shown).

These results indicate that TrwB localizes at a cell pole independently of the R388-encoded conjugative proteins.

3.3. The soluble mutant protein TrwBΔN70 needs other R388 proteins for positioning at the membrane

Many biochemical and biophysical differences between TrwB and TrwBΔN70 have been reported [29,33,34]. To add more information to the role of the TMD in the properties of TrwB, we analyzed the subcellular location of the soluble mutant protein TrwBΔN70. Interestingly, immunofluorescence images showed two different patterns for TrwB ΔN70 mutant protein. TrwBΔN70 remained in the cytosol in the

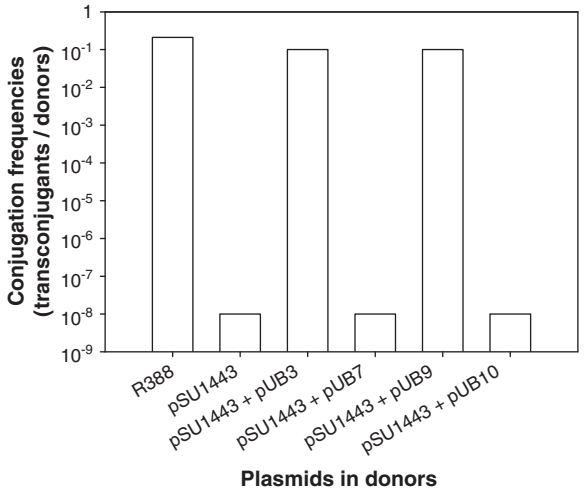
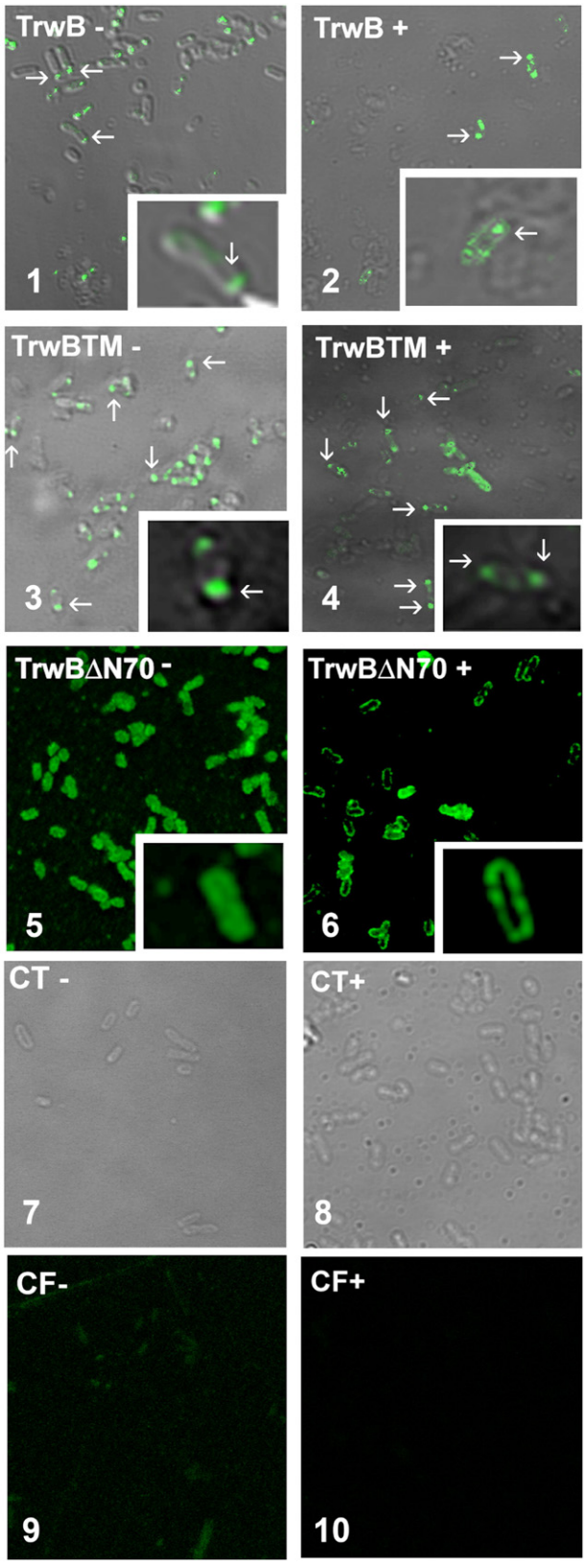


Fig. 1. Transfer frequencies of plasmid pSU1443 (*trwB*$^-$) complemented with TrwB–GFP or TrwBTM–GFP. Transfer frequencies of plasmids bearing TrwB–GFP (pUB9) or TrwBTM–GFP (pUB10) fusion proteins were evaluated by examining complementation ability in cells harboring R388 plasmid deficient in *trwB* (pSU1443). For comparison, transfer frequencies of TrwB (pUB3) and TrwBTM (pUB7) are shown in the same figure. Transfer frequencies were normalized to the number of transconjugants per donor cell. Frequencies were calculated with data from at least five independent experiments.

absence of R388 as observed by the immunolocalization images (Fig. 2, panel 5). In contrast, in the presence of the remaining conjugative proteins of R388, TrwBΔN70 appeared evenly distributed in the membrane but did not localize in discrete foci (Fig. 2, panel 6).

These results suggest that the cytosolic domain of TrwB needs its TMD for polar location in the bacteria.



3.4. The TMD of TrwB targets TrwB to the cell pole

In view of the differences found between the subcellular location of TrwB and its soluble mutant protein TrwBΔN70, we decided to visualize the subcellular location of the TMD alone. In order to do so, we analyzed the subcellular location of the TrwBTM mutant protein by immunofluorescence microscopy as previously described. Mouse anti-His (C-term) monoclonal antibody (Invitrogen) and Alexa Fluor 488 goat anti-mouse antibodies were used as primary and secondary antibodies, respectively. Immunofluorescence images showed that TrwBTM mutant protein localized at discrete foci to the cell pole (Fig. 2, panel 4) even when the remaining conjugative proteins encoded by R388 were not present (Fig. 2, panel 3).

To confirm these results we visualized the fusion protein TrwBTM–GFP by confocal microscopy. Polar foci were also observed with this technique in the absence or presence of the remaining conjugative proteins of R388 (Fig. 3, panels 4 and 8, respectively). Quantitative analysis showed that 80% of the cells presented foci and 94% of the foci were localized at a single cell pole (Table 3).

These results suggest that the polar location of TrwB is attributed to the TMD of the protein because the TrwBTM mutant protein, that only contains the TMD, can be found at the cell pole even in the absence of the remaining R388 conjugative proteins.

3.5. TrwB is preferentially localized to one cell pole independently of the growth phase of E. coli

Pole distribution of both TrwB and TrwBTM at different expression times and in different hosts was studied (Table 3 and Fig. 4). *E. coli* BL21C41 (DE3) and BL21C43 (DE3) strains were chosen because they are effective in overexpressing membrane proteins [38]. Table 3 summarizes the statistical analysis on subcellular location of TrwB and TrwBTM. It can be observed that no significant differences in distribution or in number of cells showing polar foci were observed for *E. coli* BL21C41 (DE3) and BL21C43 (DE3) strains for TrwB and TrwBTM mutant protein (Table 3 and Fig. 4). Regarding growth phase, the maximum percentage of cells showing a polar location of TrwB occurred at late exponential and early stationary growth phases (*i.e.*, 4 and 6 h after induction, respectively) (Table 3 and Fig. 5). These samples showed more than 75% of the cells with polar foci and more than 61% of those cells showed foci at a single cell pole (Table 3). At stationary growth phase (20 h) around 60% of the cells showed discrete polar foci in their membrane, both when TrwB and TrwBTM were visualized (Fig. 4). Nevertheless, again the percentage of cells showing foci at a single pole represented more than 90% of the cells with foci (Table 3). In like manner, at early stages of growth (*i.e.*, 1 h after induction) only 23% of the cells showed polar foci but all were located at a single pole as shown in other growth phases (Table 3).

4. Discussion

Conjugation systems are machines that translocate DNA substrates to recipient cells by a contact-dependent process [1]. These systems are typically composed of up to 12 proteins that take part in three different biochemical reactions, *i.e.*, DNA substrate processing, substrate recruitment, and translocation. Since this process involves the assembly

Fig. 2. Subcellular location of TrwB protein, and TrwBTM and TrwBΔN70 mutant proteins visualized by immunofluorescence microscopy (IFM). Subcellular location of TrwB, TrwBΔN70 and TrwBTM proteins was determined in *E. coli* strains containing plasmid pSU1443 that expresses all R388 conjugative proteins except *trwB* (plus symbol, right panels) or without plasmid pSU1443 (minus symbol, left panels). Cells were immunostained with either rabbit serum anti-TrwB (panels 1, 2, 5 and 6) or mouse anti-His (C-term) monoclonal antibody (Invitrogen) (panels 3 and 4). In general 60 fold magnification was used and the inserts show selected images at a higher magnification (30 fold). Control fluorescence (CF) and control transmitted light (CT) images of cells that lacked *trwB* were used as negative controls (panels 7–10). Arrowheads indicate foci at the cell pole.

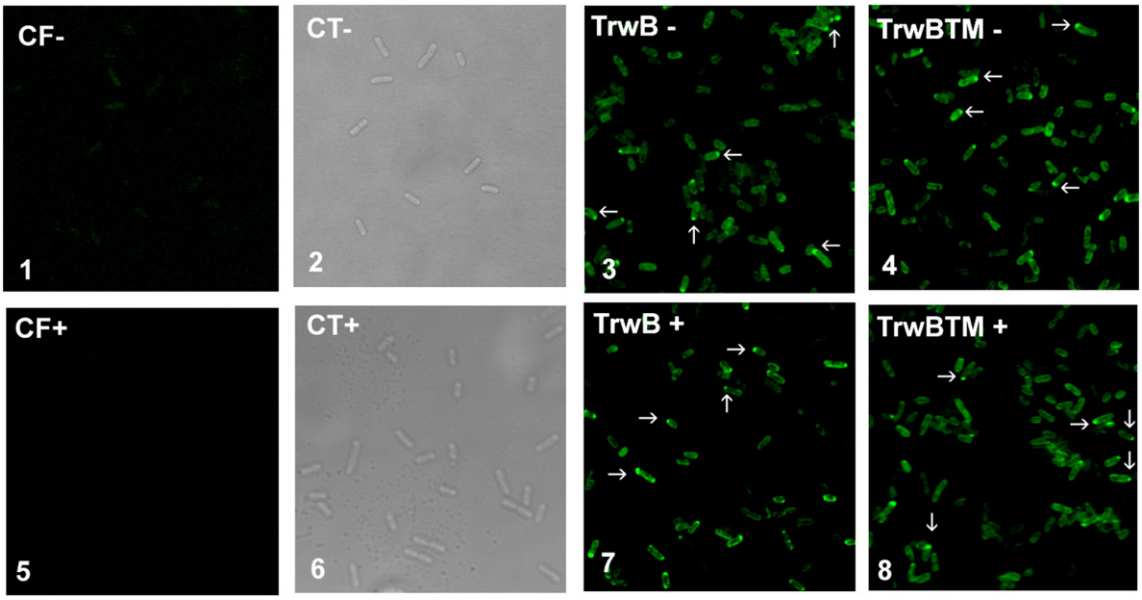


Fig. 3. Subcellular location of TrwB–GFP and TrwBTM–GFP fusion proteins. Subcellular location of cells expressing TrwB–GFP and TrwBTM–GFP proteins in *E. coli* strains containing plasmid pSU1443 that expresses all R388 conjugative proteins except *trwB* (plus symbol, bottom panels) or without pSU1443 (minus symbol, upper panels) was determined. Panels 3 and 7 correspond to cells expressing TrwB–GFP. Panels 4 and 8 correspond to cells expressing TrwBTM–GFP. Control fluorescence (CF) (panels 1 and 5) and control transmitted light (CT) (panels 2 and 6) images of cells that lacked *trwB* were used as negative controls. Arrowheads indicate foci at the cell pole.

of a multiprotein complex in the bacterial membrane, the cellular location of different conjugative proteins is of great interest to understand the function of these systems.

The ability of some conjugative proteins of different systems to localize at discrete foci within the cell membrane has been already reported [13–18,45,47,48,51]. In particular, VirB10 and VirB9 were randomly distributed in the cell membrane and became localized in clusters only when the remaining conjugative proteins were present [13]. Also, VirB3, an inner membrane protein, was able to localize to the pole but required VirB4, VirB7 and VirB8 for VirB3 stabilization [48]. Zambryski and coworkers have reported that some of these conjugative proteins are organized in a periodic pattern of foci [49,50,52].

In particular, the subcellular location of T4CPs is of great interest because these proteins are supposed to be the active link between the relaxosome and the conduit created by the T4SS. In this regard, subcellular location of T4CPs VirD4 [14] and TraG [16] has been already described. Nevertheless this remains controversial because while TraG forms membrane-associated fluorescent foci at random positions independently of R27 conjugative proteins [16], VirD4 protein localizes to the cell poles in the absence of other conjugative proteins [14]. Given these discrepancies in the literature, we decided to study the subcellular location of TrwB, the T4CP of R388 conjugative plasmid.

Immunofluorescence microscopy and GFP fusion proteins were used to test the location pattern of TrwB. Both techniques showed that TrwB localized to a cell pole even in the absence of R388 conjugative proteins. Therefore, our results are in agreement with the polar position of VirD4 [14]. Kumar and Das [14] also reported that the cytosolic NBD was essential for the polar location of VirD4. On the contrary, our studies show that the TMD of TrwB alone is sufficient for polar location of TrwB even when no other R388 proteins were present. Therefore, according to our results, polar location of TrwB could be attributed to the TMD of the protein. By contrast, the soluble mutant protein TrwBΔN70 appeared in the cytosol and only when the remaining R388 conjugative proteins were present, this mutant protein appeared uniformly distributed in the membrane but did not form foci. This result indicates that some interactions must occur between the cytosolic domain and some of the conjugative proteins encoded by R388 that would drag this soluble mutant protein towards the membrane. In sum, these results suggest that the TMD of TrwB could be the key element for its polar location and that the cytosolic domain could interact with other integral or peripheral membrane proteins of R388, such as TrwE or TrwK, as previously reported [44,46]. According to Kumar and Das the formation of foci in IFM images suggested oligomerization of the T4CP VirD4 [14]. The fact that TrwB and TrwBTM but not TrwBΔN70 formed foci also suggests that the TMD could be necessary

Table 3
Subcellular location of TrwB and TrwBTM mutant protein in different strains and at different protein expression times.

Strain (*E. coli*)	Protein	Expression time (h)	No of cells analyzed	Cells with polar location (% of total cells)	Cells with unipolar location (% of total cells)	Cells with unipolar location (% of cells with poles)[a]
BL21C41	TrwB	1	69	16 (23%)	16 (23%)	100
		4	64	54 (84%)	47 (73%)	87
		6	68	51 (75%)	44 (65%)	86
		20	61	36 (59%)	35 (57%)	97
BL21C43	TrwB	4	51	38 (75%)	31 (61%)	82
		6	47	36 (77%)	30 (64%)	83
		20	65	38 (59%)	35 (54%)	92
BL21C41	TrwBTM	20	88	70 (80%)	66 (75%)	94
BL21C43	TrwBTM	20	62	41 (66%)	38 (61%)	93

[a] The remaining cells have bipolar location.

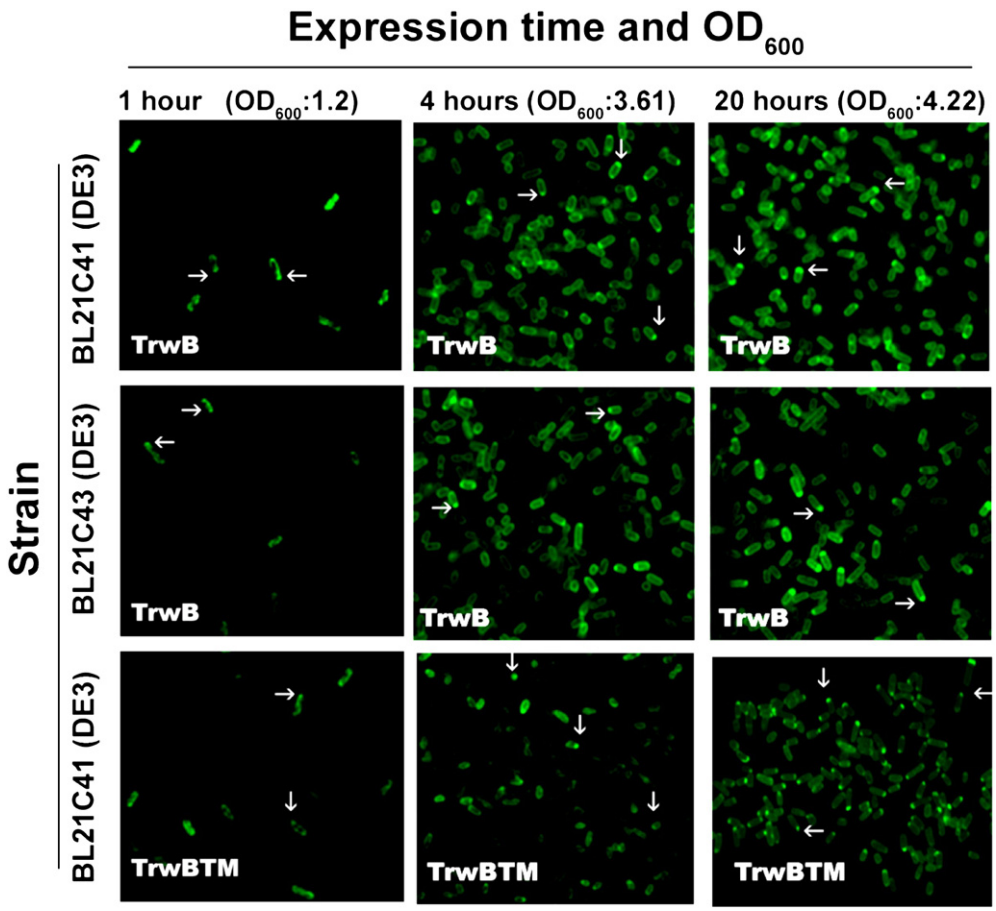


Fig. 4. Subcellular location of TrwB and TrwBTM using GFP-fusion proteins at different expression times and in different *E. coli* strains. After induction with 1 mM IPTG cells were incubated at 25 °C for 1, 4 or 20 h. Fluorescence confocal images were taken with a 60× oil immersion objective, using an excitation wavelength of 488 nm. The emission was recovered between 505–525 nm. Arrowheads indicate foci at cell pole.

to form TrwB oligomers at the cell pole of the bacterium. This result is in agreement with the role of the TMD of TrwB in oligomerization previously reported by our group [27,46].

When the amino acid sequence of the TMD was analyzed, no special motifs that explain its role in intracellular location were found. Protein

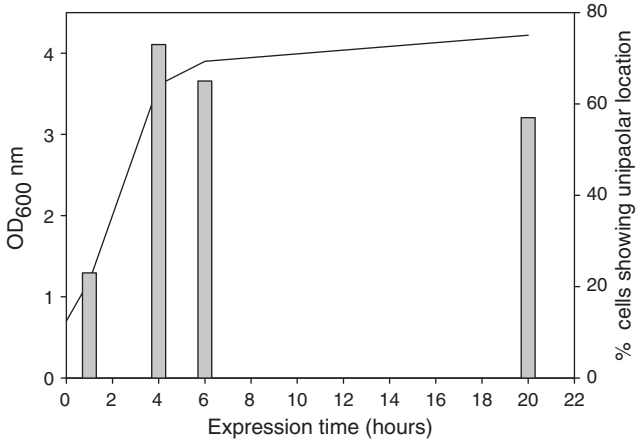


Fig. 5. Location of TrwB in relation to the growth phase. Number of cells showing TrwB–GFP foci at different growth phases of *E. coli* BL21C41 cells transformed with plasmid pUB9 containing *trwB–GFP* gene. Continuous line represents OD_{600} values and grey bars represent percentage of cells showing unipolar location.

polar location could be mediated by complex and dynamic changes in cellular transduction, cytoskeleton proteins or specific regions of the chromosome [53]. The location of membrane proteins at cell poles has been also related to the presence of cardiolipin-rich domains that modify membrane curvature [54,55]. But in particular, the polar location of TrwB could be interpreted in the light of our knowledge of the Stb system of plasmid R388, in the crosstalk between conjugation and plasmid stability [56]. In fact, *stbA* deletion mutants of R388 localize the plasmid copies to the cell poles, increasing conjugation frequencies by up to 50-fold as a result of an improved access of the plasmid copies to the conjugative transport channel [56].

When growth-dependent TrwB pole distribution was analyzed it was observed that 84% of early exponential-phase cells showed TrwB–GFP location at the cell poles and 73% of the cells showed unipolar location. The number of cells showing poles was constant during late exponential and late stationary phases in the absence of other conjugative proteins (Fig. 5).

Our previous studies reported the effect of TMD of TrwB on its transfer capacity [46], nucleotide binding activity [29], stability and secondary structure [27,34,35] and oligomerization [27,46]. As a conclusion, in the present work the important role of the TMD in the cellular location of TrwB is described. The fact that TrwB and, more importantly the TMD alone, but not the soluble mutant protein TrwBΔN70, localize to the cell poles suggests that the TMD of TrwB is a key element that governs the polar location of TrwB. In sum, the present work contributes to underscore the coupling role of T4CPs that link the relaxosome with the conjugative transport channel in the conjugative process.

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References

[1] C.E. Alvarez-Martinez, P.J. Christie, Biological diversity of prokaryotic type IV secretion systems, Microbiol. Mol. Biol. Rev. 73 (2009) 775–808.
[2] F. Baquero, T.M. Coque, F. de la Cruz, Ecology and evolution as targets: the need for novel eco-evo drugs and strategies to fight antibiotic resistance, Antimicrob. Agents Chemother. 55 (2011) 3649–3660.
[3] E.L. Zechner, S. Lang, J.F. Schildbach, Assembly and mechanisms of bacterial type IV secretion machines, Philos. Trans. R. Soc. Lond. B Biol. Sci. 367 (2012) 1073–1087.
[4] O. Draper, C.E. César, C. Machón, F. de la Cruz, M. Llosa, Site-specific recombinase and integrase activities of a conjugative relaxase in recipient cells, Proc. Natl. Acad. Sci. U. S. A. 102 (2005) 16385–16390.
[5] C.E. César, C. Machón, F. de la Cruz, M. Llosa, A new domain of conjugative relaxase TrwC responsible for efficient *oriT*-specific recombination on minimal target sequences, Mol. Microbiol. 62 (2006) 984–996.
[6] M.P. Garcillan-Barcia, M.V. Francia, F. de la Cruz, The diversity of conjugative relaxases and its application in plasmid classification, FEMS Microbiol. Rev. 33 (2009) 657–687.
[7] E. Cabezon, F. de la Cruz, TrwB: an F(1)-ATPase-like molecular motor involved in DNA transport during bacterial conjugation, Res. Microbiol. 157 (2006) 299–305.
[8] K. Wallden, A. Rivera-Calzada, G. Waksman, Type IV secretion systems: versatility and diversity in function, Cell. Microbiol. 12 (2010) 1203–1212.
[9] L. Terradot, R. Bayliss, C. Oomen, G.A. Leonard, C. Baron, G. Waksman, Structures of two core subunits of the bacterial type IV secretion system, VirB8 from *Brucella suis* and ComB10 from *Helicobacter pylori*, Proc. Natl. Acad. Sci. U. S. A. 102 (2005) 4596–4601.
[10] R. Bayliss, R. Harris, L. Coutte, A. Monier, R. Fronzes, P.J. Christie, P.C. Driscoll, G. Waksman, NMR structure of a complex between the VirB9/VirB7 interaction domains of the pKM101 type IV secretion system, Proc. Natl. Acad. Sci. U. S. A. 104 (2007) 1673–1678.
[11] V. Chandran, R. Fronzes, S. Duquerroy, N. Cronin, J. Navaza, G. Waksman, Structure of the outer membrane complex of a type IV secretion system, Nature 462 (2009) 1011–1015.
[12] R. Fronzes, E. Schäfer, L. Wang, H.R. Saibil, E.V. Orlova, G. Waksman, Structure of a type IV secretion system core complex, Science 323 (2009) 266–268.
[13] R.B. Kumar, Y.H. Xie, A. Das, Subcellular localization of the *Agrobacterium tumefaciens* T-DNA transport pore proteins: VirB8 is essential for the assembly of the transport pore, Mol. Microbiol. 36 (2000) 608–617.
[14] R.B. Kumar, A. Das, Polar location and functional domains of the *Agrobacterium tumefaciens* DNA transfer protein VirD4, Mol. Microbiol. 43 (2002) 1523–1532.
[15] K. Atmakuri, Z. Ding, P.J. Christie, VirE2, a type IV secretion substrate, interacts with the VirD4 transfer protein at cell poles of *Agrobacterium tumefaciens*, Mol. Microbiol. 49 (2003) 1699–1713.
[16] J.E. Gunton, M.W. Gilmour, G. Alonso, D.E. Taylor, Subcellular localization and functional domains of the coupling protein, TraG, from IncHI1 plasmid R27, Microbiology 151 (2005) 3549–3561.
[17] P.K. Judd, R.B. Kumar, A. Das, The type IV secretion apparatus protein VirB6 of *Agrobacterium tumefaciens* localizes to a cell pole, Mol. Microbiol. 55 (2005) 115–124.
[18] P.K. Judd, R.B. Kumar, A. Das, Spatial location and requirements for the assembly of the *Agrobacterium tumefaciens* type IV secretion apparatus, Proc. Natl. Acad. Sci. U. S. A. 102 (2005) 11498–11503.
[19] M.W. Gilmour, J.E. Gunton, T.D. Lawley, D.E. Taylor, Interaction between the IncHI1 plasmid R27 coupling protein and type IV secretion system: TraG associates with the coiled-coil mating pair formation protein TrhB, Mol. Microbiol. 49 (2003) 105–116.
[20] F.X. Gomis-Rüth, F. de la Cruz, M. Coll, Structure and role of coupling proteins in conjugal DNA transfer, Res. Microbiol. 153 (2002) 199–204.
[21] M. Llosa, F. de la Cruz, Bacterial conjugation: a potential tool for genetic engineering, Res. Microbiol. 156 (2005) 1–6.
[22] A. Das, Y.H. Xie, Construction of transposon Tn3phoA: its application in defining the membrane topology of the *Agrobacterium tumefaciens* DNA transfer proteins, Mol. Microbiol. 27 (1998) 405–414.
[23] G. Schröder, S. Krause, E.L. Zechner, B. Traxler, H.J. Yeo, R. Lurz, G. Waksman, E. Lanka, TraG-like proteins of DNA transfer systems and of the *Helicobacter pylori* type IV secretion system: inner membrane gate for exported substrates? J. Bacteriol. 184 (2002) 2767–2779.
[24] G. Moncalián, E. Cabezón, I. Alkorta, M. Valle, F. Moro, J.M. Valpuesta, F.M. Goñi, F. de la Cruz, Characterization of ATP and DNA binding activities of TrwB, the coupling protein essential in plasmid R388 conjugation, J. Biol. Chem. 274 (1999) 36117–36124.
[25] F.X. Gomis-Rüth, G. Moncalián, R. Pérez-Luque, A. González, E. Cabezón, F. de la Cruz, M. Coll, The bacterial conjugation protein TrwB resembles ring helicases and F1-ATPase, Nature 409 (2001) 637–641.
[26] F.X. Gomis-Rüth, G. Moncalián, F. Cruz, M. Coll, Conjugative plasmid protein TrwB, an integral membrane type IV secretion system coupling protein. Detailed structural features and mapping of the active site cleft, J. Biol. Chem. 277 (2002) 7556–7566.
[27] I. Hormaeche, I. Alkorta, F. Moro, J.M. Valpuesta, F.M. Goñi, F. de la Cruz, Purification and properties of TrwB, a hexameric, ATP-binding integral membrane protein essential for R388 plasmid conjugation, J. Biol. Chem. 277 (2002) 46456–46462.
[28] I. Tato, S. Zunzunegui, F. de la Cruz, E. Cabezon, TrwB, the coupling protein involved in DNA transport during bacterial conjugation, is a DNA-dependent ATPase, Proc. Natl. Acad. Sci. U. S. A. 102 (2005) 8156–8161.
[29] I. Hormaeche, R.L. Segura, A.J. Vecino, F.M. Goñi, F. de la Cruz, I. Alkorta, The transmembrane domain provides nucleotide binding specificity to the bacterial conjugation protein TrwB, FEBS Lett. 580 (2006) 3075–3082.
[30] I. Tato, I. Matilla, I. Arechaga, S. Zunzunegui, F. de la Cruz, E. Cabezon, The ATPase activity of the DNA transporter TrwB is modulated by protein TrwA: implications for a common assembly mechanism of DNA translocating motors, J. Biol. Chem. 282 (2007) 25569–25576.
[31] I. Matilla, C. Alfonso, G. Rivas, E.L. Bolt, F. de la Cruz, E. Cabezon, The conjugative DNA translocase TrwB is a structure-specific DNA-binding protein, J. Biol. Chem. 285 (2010) 17537–17544.
[32] A.J. Vecino, R.L. Segura, B. Ugarte-Uribe, S. Aguila, I. Hormaeche, F. de la Cruz, F.M. Goñi, I. Alkorta, Reconstitution in liposome bilayers enhances nucleotide binding affinity and ATP-specificity of TrwB conjugative coupling protein, Biochim. Biophys. Acta 1798 (2010) 2160–2169.
[33] I. Hormaeche, I. Iloro, J.L. Arrondo, F.M. Goñi, F. de la Cruz, I. Alkorta, Role of the transmembrane domain in the stability of TrwB, an integral protein involved in bacterial conjugation, J. Biol. Chem. 279 (2004) 10955–10961.
[34] A.J. Vecino, I. de la Arada, R.L. Segura, F. de la Cruz, F.M. Goñi, I. Alkorta, Membrane insertion stabilizes the structure of TrwB the R388 conjugative plasmid coupling protein, Biochim. Biophys. Acta 1808 (2011) 1032–1039.
[35] A.J. Vecino, R.L. Segura, I. de la Arada, F. Cruz, F.M. Goñi, J.L.R. Arrondo, I. Alkorta, Deletion of a single helix from the transmembrane domain causes large changes in membrane insertion properties and secondary structure of the bacterial conjugation protein TrwB, Biochim. Biophys. Acta 1818 (2012) 3158–3166.
[36] S.G. Grant, J. Jessee, F.R. Bloom, D. Hanahan, Differential plasmid rescue from transgenic mouse DNAs into *Escherichia coli* methylation-restriction mutants, Proc. Natl. Acad. Sci. U. S. A. 87 (1990) 4645–4649.
[37] F. de la Cruz, J. Grinsted, Genetic and molecular characterization of Tn21, a multiple resistance transposon from R100.1, J. Bacteriol. 151 (1982) 222–228.
[38] B. Miroux, J.E. Walker, Over-production of proteins in *Escherichia coli*: mutant hosts that allow synthesis of some membrane proteins and globular proteins at high levels, J. Mol. Biol. 260 (1996) 289–298.
[39] F.W. Studier, B.A. Moffatt, Use of bacteriophage T7 RNA polymerase to direct selective high-level expression of cloned genes, J. Mol. Biol. 189 (1986) 113–130.
[40] G.S. Waldo, B.M. Standish, J. Berendzen, T.C. Terwilliger, Rapid protein-folding assay using green fluorescent protein, Nat. Biotechnol. 17 (1999) 691–695.
[41] N. Datta, R.W. Hedges, Trimethoprim resistance conferred by W plasmids in *Enterobacteriaceae*, J. Gen. Microbiol. 72 (1972) 349–355.
[42] M. Llosa, S. Bolland, F. de la Cruz, Genetic organization of the conjugal DNA processing region of the IncW plasmid R388, J. Mol. Biol. 235 (1994) 448–464.
[43] J. Sambrook, E.F. Fritsch, T. Maniatis, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Lab. Press, Plainview, New York, 1989.
[44] M. Llosa, S. Zunzunegui, F. de la Cruz, Conjugative coupling proteins interact with cognate and heterologous VirB10-like proteins while exhibiting specificity for cognate relaxosomes, Proc. Natl. Acad. Sci. U. S. A. 100 (2003) 10465–10470.
[45] W.L. Teng, T.L. Bannam, J.A. Parsons, J.I. Rood, Functional characterization and localization of the TcpH conjugation protein from *Clostridium perfringens*, J. Bacteriol. 190 (2008) 5075–5086.
[46] R.L. Segura, S. Águila-Arcos, B. Ugarte-Uribe, A.J. Vecino, F. de la Cruz, F.M. Goñi, I. Alkorta, The transmembrane domain of the T4SS coupling protein TrwB and its role in protein–protein interactions, Biochim. Biophys. Acta 1828 (2013) 2015–2025.
[47] T. Bauer, T. Rösch, M. Itaya, P.L. Graumann, Localization pattern of conjugation machinery in a gram-positive bacterium, J. Bacteriol. 193 (2011) 6244–6256.
[48] P. Mossey, A. Hudacek, A. Das, *Agrobacterium tumefaciens* type IV secretion protein VirB3 is an inner membrane protein and requires VirB4, VirB7, and VirB8 for stabilization, J. Bacteriol. 192 (2010) 2830–2838.
[49] J. Aguilar, T.A. Cameron, J. Zupan, P. Zambryski, Membrane and core periplasmic *Agrobacterium tumefaciens* virulence Type IV secretion system components localize to multiple sites around the bacterial perimeter during lateral attachment to plant cells, MBio 2 (2011) e00218-11.
[50] T.A. Cameron, M. Roper, P. Zambryski, Quantitative image analysis and modeling indicate the *Agrobacterium tumefaciens* type IV secretion system is organized in a periodic pattern of foci, PLoS One 7 (2012) e42219.

[51] K. Atmakuri, E. Cascales, O.T. Burton, L.M. Banta, P.J. Christie, *Agrobacterium* ParA/MinD-like VirC1 spatially coordinates early conjugative DNA transfer reactions, EMBO J. 26 (2007) 2540–2551.
[52] J. Aguilar, J. Zupan, T.A. Cameron, P.C. Zambryski, *Agrobacterium* type IV secretion system and its substrates form helical arrays around the circumference of virulence-induced cells, Proc. Natl. Acad. Sci. U. S. A. 107 (2010) 3758–3763.
[53] L. Shapiro, H.H. McAdams, R. Losick, Generating and exploiting polarity in bacteria, Science 298 (2002) 1942–1946.
[54] A. Nishibori, J. Kusaka, H. Hara, M. Umeda, K. Matsumoto, Phosphatidylethanolamine domains and localization of phospholipid synthases in *Bacillus subtilis* membranes, J. Bacteriol. 187 (2005) 2163–2174.
[55] T. Romantsov, S. Helbig, D.E. Culham, C. Gill, L. Stalker, J.M. Wood, Cardiolipin promotes polar localization of osmosensory transporter ProP in *Escherichia coli*, Mol. Microbiol. 64 (2007) 1455–1465.
[56] C. Guynet, A. Cuevas, G. Moncalián, F. de la Cruz, The stb operon balances the requirements for vegetative stability and conjugative transfer of plasmid R388, PLoS Genet. 7 (2011) e1002073.