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The ATPase Activity of the DNA Transporter TrwB Is Modulated by Protein TrwA

*IMPLICATIONS FOR A COMMON ASSEMBLY MECHANISM OF DNA TRANSLOCATING MOTORS**

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Conjugative systems contain an essential integral membrane protein involved in DNA transport called the Type IV coupling protein (T4CP). The T4CP of conjugative plasmid R388 is TrwB, a DNA-dependent ATPase. Biochemical and structural data suggest that TrwB uses energy released from ATP hydrolysis to pump DNA through its central channel by a mechanism similar to that used by F_1-ATPase or ring helicases. For DNA transport, TrwB couples the relaxosome (a DNA-protein complex) to the secretion channel. In this work we show that TrwA, a tetrameric *oriT* DNA-binding protein and a component of the R388 relaxosome, stimulates TrwBΔN70 ATPase activity, revealing a specific interaction between the two proteins. This interaction occurs via the TrwA C-terminal domain. A 68-kDa complex between TrwBΔN70 and TrwA C-terminal domain was observed by gel filtration chromatography, consistent with a 1:1 stoichiometry. Additionally, electron microscopy revealed the formation of oligomeric TrwB complexes in the presence, but not in the absence, of TrwA protein. TrwBΔN70 ATPase activity in the presence of TrwA was further enhanced by DNA. Interestingly, maximal ATPase rates were achieved with TrwA and different types of dsDNA substrates. This is consistent with a role of TrwA in facilitating the interaction between TrwB and DNA. Our findings provide a new insight into the mechanism by which TrwB recruits the relaxosome for DNA transport. The process resembles the mechanism used by other DNA-dependent molecular motors, such as the RuvA/RuvB system, to be targeted to the DNA followed by hexamer assembly.

Conjugative DNA transfer is an efficient way for bacteria to acquire new genetic information. Any DNA molecule containing a short segment called origin of transfer (*oriT*)[4] can be conjugatively transferred to a recipient cell if a conjugation machinery is provided. This machinery consists of two functional subsets of proteins: Dtr proteins (involved in the processing of conjugative DNA) and Mpf proteins (involved in the production of exocellular pili and the formation of a channel structure for secretion). Both protein subsets are encoded by the transfer region of a conjugative plasmid (1).

R388 plasmid displays one of the simplest transfer regions in Gram-negative bacteria, coding for just three Dtr proteins: TrwA, TrwB, and TrwC. TrwC is a two domain (relaxase and DNA helicase) protein. Its relaxase domain introduces a site-specific nick at *oriT* and remains covalently bound to the 5′-end of the DNA strand that is transferred. Therefore, TrwC acts as a pilot protein in the transfer of the resulting nucleoprotein complex. Recent work demonstrated that TrwC still plays an essential role in the recipient cell, where it performs the strand-transfer reaction required to recircularize the transferred DNA and thus, terminates conjugation (2, 3). The atomic structure of the relaxase domain of TrwC was solved (4), showing a fold shared by the relaxase of plasmid F (5) and replication initiation proteins of some viruses (6, 7). TrwA is a tetrameric *oriT*-specific DNA-binding protein that belongs to the ribbon-helix-helix protein family DNA-binding proteins (8). Experimental data revealed that TrwA binds to two specific sites in R388 *oriT*: *sbaA* and *sbaB*. Binding to these sites enhances TrwC relaxase activity and represses *trwABC* operon transcription (9). TrwA consists of two structural domains: an N-terminal domain, involved in DNA binding and a C-terminal domain, responsible for TrwA tetramerization (8). Recently, the structure of the C-terminal domain of the related protein TraM from plasmid F was solved (10). The nucleoprotein complex formed by *oriT*, relaxase TrwC, host protein IHF, and protein TrwA is called the relaxosome, which must be recruited to the secretion channel for DNA transfer. The third Dtr protein, TrwB, is an inner membrane protein that couples the relaxosome to a type IV secretion system (T4SS), a multiprotein complex that spans the inner and outer membranes. In R388 plasmid, the T4SS consists of eleven proteins (TrwD to TrwN). Direct interactions between TrwB and TrwE, an inner membrane protein, were described (11) and corroborated with protein homologues in IncH plasmids (12). On the cytoplasmic side, the coupling protein interacts

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[4] The abbreviations used are: *oriT*, origin of transfer; PMSF, phenylmethylsulfonyl fluoride; Pipes, 1,4-piperazinediethanesulfonic acid; ssDNA, single-stranded DNA; dsDNA, double-stranded DNA.

with relaxosomal components. In R388, interactions between TrwB and the relaxosome occur via TrwA and TrwC (11). In IncF plasmids, interactions between the coupling protein TraD and TraM have also been reported (13, 14). Besides its coupling role in bacterial conjugation, TrwB is also thought to work actively as a DNA transporter. The crystallographic structure of TrwBΔN70, the cytoplasmic domain of TrwB, revealed a hexamer with 6-fold symmetry and a central channel of about 20 Å in diameter (15). The striking structural similarity between TrwB and other well known molecular motors, such as F_1-ATPase (16) or T7 gene 4 helicase (17), suggested that TrwB might also operate as a motor, pumping DNA through its central channel using energy derived from ATP hydrolysis. This hypothesis is strengthened by recent biochemical data, which demonstrate that TrwB is a DNA-dependent ATPase (18). The protein assembles as an oligomer on ssDNA and exhibits positive cooperativity for ATP hydrolysis. Following this idea, a mechanism similar to the so-called "binding change mechanism" assigned to F_1-ATPase, was proposed for TrwB (19).

In this work, we have extended the analysis of TrwB ATPase activity by using different DNA substrates. We have also observed that TrwB ATPase activity is stimulated by protein TrwA, revealing specific interactions between the two proteins. This modulation might facilitate the dynamic coupling between TrwB and DNA during the conjugative process. These findings shed light on the coupling role assigned to TrwB (20) and on the mechanism by which this protein recruits the relaxosome for conjugative transport. The system resembles the mechanism used by other DNA-dependent molecular motors, such as the RuvA/RuvB recombination system (21), to be targeted and assembled as hexamers on the DNA, leading us to propose a common mechanism of assembly for hexameric molecular motors.

MATERIALS AND METHODS

Protein Purification and Preparation of DNA Substrates—Purification of protein TrwBΔN70 was carried out as described previously (18). TrwA-derived proteins containing a C-terminal His tag were purified as follows. Strains used were derivatives of *Escherichia coli* C41 (22) containing plasmids pSU1547, pSU1548, and pSU1550, which overexpress TrwA-His_6, TrwA-(2–73)-His_6 and TrwA-(36–121)-His_6, respectively (8). Bacteria were grown in 2 liters of LB medium. After addition of isopropyl-1-thio-β-D-galactopyranoside (Apollo), cells were incubated for 8 h at 37 °C, harvested, and resuspended in 40 ml of buffer A (100 mM Tris-HCl, pH 7, 500 mM NaCl, 0.001% PMSF) supplemented with 2.5 mM benzamidine. Cells were lysed by sonication (Vibra Cell, Sonic & Materials Inc.), and the lysate was centrifuged at 138,000 × *g* for 30 min at 4 °C. The supernatant was then loaded to a His trap HP (Amersham Biosciences) chromatographic column, equilibrated with buffer A. Bound proteins were eluted in a 50-ml imidazole gradient (0–500 mM) with buffer B (100 mM Tris-HCl, pH 7, 200 mM NaCl, 500 mM imidazole, 0.001% PMSF). TrwA-containing fractions were pooled and concentrated in a Centricon YM10 (Amicon) concentrator to 4 ml. Then, protein was loaded on a HiLoad 16/60 Superdex 75 column (Amersham Biosciences) equilibrated with buffer 50 mM Pipes-NaOH, pH 6.2, 100 mM NaCl, 2 mM $MgCl_2$, 0.1 mM EDTA, 10% glycerol and 0.001% PMSF. Protein was stored at 4 °C. Protein concentrations were determined by the method of Bradford (Bio-Rad).

Circular DNA substrates, obtained by using Qiagen plasmid DNA purification kit, were vector plasmid pUC8 (23) and pSU1186 (24), which contains R388 *oriT* cloned in pUC8. Additionally, a linear DNA fragment corresponding to nucleotides 15934–16622 from the R388 *oriT* DNA sequence (GenBank™ accession no BR000038) was obtained by PCR amplification with appropriate oligonucleotides. Circular ssM13DNA was prepared as described in Ref. 25. The 45-mer oligonucleotide used (5′-TCG CCA CGT TTC GCC GTT TGC GGG GGT TTC TGC GAG GAA CTT TGG-3′) was described previously (18). ssDNA and dsDNA concentrations of the stock solutions were measured by absorbance at 260 nm, using 40 $\mu g \cdot ml^{-1} \cdot A_{260}{}^{-1}$ and 50 $\mu g \cdot ml^{-1} \cdot A_{260}{}^{-1}$, respectively, as conversion factors.

Nucleotide Hydrolysis Assays—ATP hydrolysis was analyzed by a coupled enzyme assay as described previously (18). To analyze the effect of increasing concentrations of ssDNA and dsDNA or the effect of TrwA-derived proteins on TrwBΔN70 ATPase activity, TrwBΔN70 was preincubated with the different substrates for 10 min at 37 °C in 150 μl of ATPase assay mixture, consisting of 50 mM Pipes-NaOH, pH 6.2, 75 mM NaCl, 6 mM MgCl, 10% glycerol, 0.5 mM phosphoenolpyruvate, 0.25 mM NADH, 60 μg/ml pyruvate kinase, and 60 μg/ml lactate dehydrogenase (Roche Applied Science). Reactions were started by addition of ATP, unless otherwise indicated.

Gel Filtration Analysis—Protein samples were chromatographed at 20 °C at a flow rate of 30 μl/min on a Superdex-200 PC 3.2/30 column (Amersham Biosciences SMART system). The column was equilibrated in buffer containing 50 mM Pipes-NaOH pH 6.2, 100 mM NaCl, 2 mM $MgCl_2$, 0.1 mM EDTA, 10% glycerol, and 0.001% PMSF. Absorbance was monitored at 280 nm. Samples (40 μl) of TrwBΔN70 (208 μg), TrwA-(36–121)-His_6 (343 μg), and the mixture of both proteins were analyzed. A 2-μl sample from each fraction was run on a 15% SDS-polyacrylamide gel, and proteins were detected by Coomassie Blue staining.

Electron Microscopy and Image Analysis—TrwBΔN70 (60 μM) was preincubated with the 45-mer oligonucleotide and chromatographed on a Superdex-200 PC 3.2/30 at 20 °C, as described previously (18). A 5-μl sample from a fast-migrating TrwBΔN70-DNA complex, with an estimated molecular mass of 440 kDa, was taken for electron microscopy. For the analysis of TrwB-TrwA complexes, TrwBΔN70 (208 μg) and TrwA-(36–121)-His_6 (343 μg) proteins were incubated for 10 min at room temperature in buffer 50 mM Pipes-NaOH pH 6.2, 100 mM NaCl, 2 mM $MgCl_2$, 0.1 mM EDTA, and 10% glycerol. Then, the sample was diluted in buffer to a protein concentration of 0.01 mg/ml and, immediately, samples were taken for the analysis.

Aliquots (5 μl) were applied to glow-discharged carbon-coated grids and stained for 1 min with 2% uranyl acetate. Images were recorded on Kodak SO163 films at ×60,000 nominal magnification in a JEOL 1200EX-II electron microscope operated at 100 KV. Micrographs were digitized in a Zeiss SCAI scanner with a final sampling rate of 2.33 Å/pixel. A total of

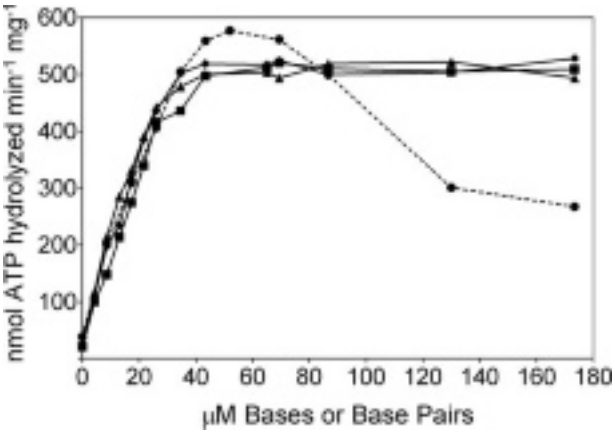

FIGURE 1. **Effect of ssDNA and dsDNA on TrwBΔN70 ATPase activity.** Reactions contained TrwBΔN70 (3 μM as monomer), 5 mM ATP, and increasing concentrations of ssDNA or dsDNA substrates, represented as micromolar concentrations of bases (for ssDNA) or base pairs (for dsDNA). (●) ssM13 DNA, (♦) PCR fragment of 688 bp, (▲) pUC8 vector, (■) plasmid pSU1186. Reactions were carried out with the coupled spectrophotometric assay. Each figure represents the average of three different experiments.

2,463 TrwB-DNA particles (or 776 particles in the case of TrwB-TrwA complexes) were selected and normalized using XMIPP image processing software (26). Alignment and classification was performed by maximum likelihood multi-reference refinement methods (27). Kernel probability density estimator self-organizing maps (28) were used for further classification of particles. Final classes consisted of averages of 150–200 particles.

RESULTS

TrwBΔN70 ATPase Activity Is Stimulated by Both ssDNA and dsDNA—In an earlier work, we characterized a DNA-dependent ATPase activity of TrwBΔN70 on ssDNA substrates, such as circular M13 ssDNA or oligonucleotides (18). In this study, we have further characterized TrwB ATPase activity by using dsDNA substrates with different DNA topologies (circular supercoiled and relaxed forms as well as linear dsDNA). Results show that ssDNA and dsDNA both stimulate TrwBΔN70 ATP hydrolysis (Fig. 1). Both types of substrates lead to similar V_{max} values (~500 nmol min^{-1} mg^{-1}). When acting on ssDNA substrates, TrwB ATPase activity showed a dependence on the absolute DNA concentration (hydrolysis rates reached a maximum and, at higher ssDNA concentrations, started to decline) (18). Fig. 1 compares this result with those obtained when using dsDNA with different topologies. Interestingly, the inhibitory effect at high DNA concentration was not observed with dsDNA substrates.

Some sequence-dependent DNA translocases, such as FtsK, move along DNA responding to directing DNA sequences (29). Additionally, some hexameric replicative helicases, such as E1 from papillomavirus, also show specificity for DNA binding at the viral origin (30). Therefore, we decided to check for DNA sequence dependence in TrwB ATPase activity. Because conjugative DNA processing starts at a specific sequence (*oriT*), we used different dsDNA substrates containing or lacking the *oriT* sequence: cloning vector pUC8, plasmid pSU1186 (R388 *oriT* cloned into pUC8) and M13 dsDNA. No difference in the kinetics of ATP hydrolysis was observed between the three dsDNA substrates (Fig. 1). Closed circular supercoiled and relaxed DNA substrates were used to check a putative dependence on DNA topology. Both types of substrates were as efficient as the 688-bp linear dsDNA fragment obtained by PCR (data not shown).

TrwA Stimulates the ATPase Activity of TrwBΔN70—TrwB has a role in recognizing the conjugative relaxosome through specific protein-protein interactions (20). The protein is anchored in the inner membrane; therefore, specific binding of TrwB to the relaxosome can position the nucleoprotein complex at the membrane interface of the transport machinery. We therefore examined whether interactions with the conjugative proteins forming the relaxosome of R388 plasmid TrwA and TrwC had any effect on TrwB ATPase activity.

TrwA-His_6 alone did not show ATPase activity either in the presence or absence of DNA (data not shown). TrwBΔN70 protein alone had a weak ATPase activity of about 30 nmol min^{-1} mg^{-1}, as described previously (18), which increased to 550 nmol min^{-1} mg^{-1} upon the addition of ssDNA. We first examined whether TrwA-His_6 had any effect on the ATPase activity of TrwBΔN70. The effect of varying the concentration of TrwA-His_6 on the rates of ATP hydrolysis in the absence of DNA and at a fixed concentration of TrwBΔN70 is shown in Fig. 2*A*. At a fixed concentration of TrwBΔN70 (0.3 μM monomer), ATP hydrolysis rates raise with increasing concentrations of TrwA, reaching a maximum of 1,300 nmol min^{-1} mg^{-1} at 0.2 μM TrwA-His_6 tetrameric protein. Above this protein concentration, TrwBΔN70 ATPase activity did not increase further. Similar experiments were carried out with TrwC. For these experiments we used the relaxase domain of TrwC, (TrwC-N293), which does not present ATPase activity. TrwC-N293 did not stimulate TrwBΔN70 ATPase activity under any of the conditions tested (data not shown).

Fig. 2*B* shows the time course of TrwBΔN70 mediated ATP hydrolysis in the presence of 0.2 μM TrwA-His_6 tetrameric protein. Reactions were initiated either by the addition of TrwBΔN70 protein or ATP. In the latter case, TrwBΔN70 and TrwA-His_6 proteins were incubated in the reaction buffer for 10 min, prior to the addition of ATP. Results show that steady-state rates of ATP hydrolysis are comparable in both cases. However, there was an evident lag phase when both proteins were not preincubated together. In that case, the time course was nonlinear for the first 10 min, suggesting that direct TrwA-TrwB protein interactions are required to observe linear progress of ATP hydrolysis.

TrwA Is More Effective in Enhancing TrwBΔN70 ATPase Activity in the Presence of DNA—A kinetic analysis of TrwBΔN70-mediated ATP hydrolysis in the presence of TrwA-His_6 protein was carried out with different DNA substrates, with results summarized in Fig. 3. Addition of both TrwA-His_6 and DNA greatly enhanced TrwBΔN70 ATPase activity. Different DNA substrates were tested for their efficiency as cofactors in the enhancement of the TrwBΔN70 ATPase activity by TrwA-His_6. In all cases, ATP hydrolysis values were stimulated to a much higher extent than in the absence of TrwA-His_6 protein (see Fig. 1 for comparison). At a fixed concentration of TrwBΔN70 (0.3 μM TrwBΔN70 monomer), the ATPase activity was enhanced about 10-fold

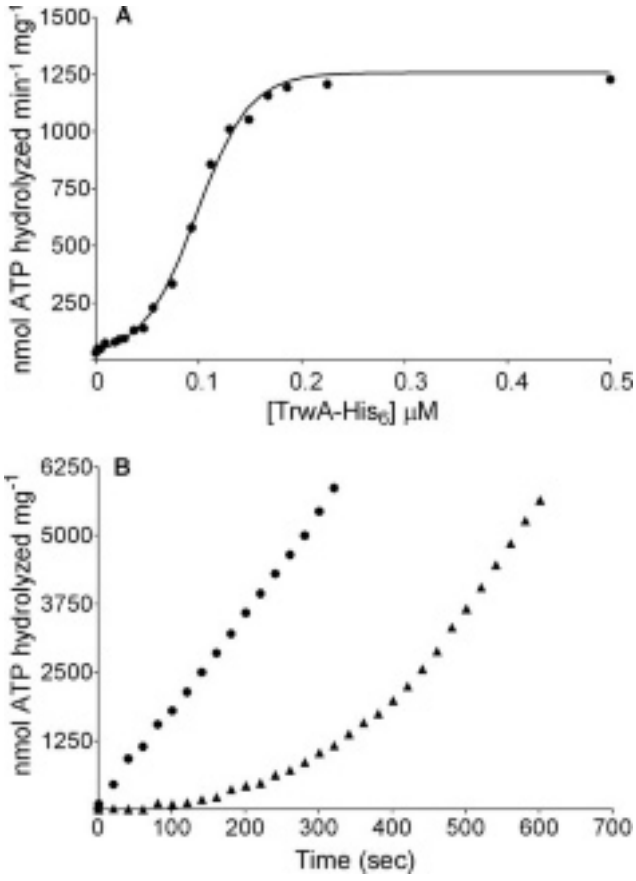

FIGURE 2. **Effect of TrwA-His$_6$ protein on TrwBΔN70-mediated ATP hydrolysis in the absence of DNA.** *A* shows the dependence of TrwBΔN70 ATP hydrolysis on TrwA-His$_6$ concentration. Reactions contained TrwBΔN70 (0.3 μM as monomer) and increasing concentrations of TrwA-His$_6$. Reactions were carried out with the coupled spectrophotometric assay, starting the reaction by the addition of 5 mM ATP, final concentration. *B* shows the time course of TrwBΔN70 protein-mediated ATP hydrolysis in the presence of 0.2 μM tetrameric TrwA-His$_6$. Reactions were carried out either by preincubation of the two proteins for 10 min, initiating the reaction by the addition of ATP (●) or without preincubation, initiating the reaction by the addition of TrwBΔN70 protein (▲).

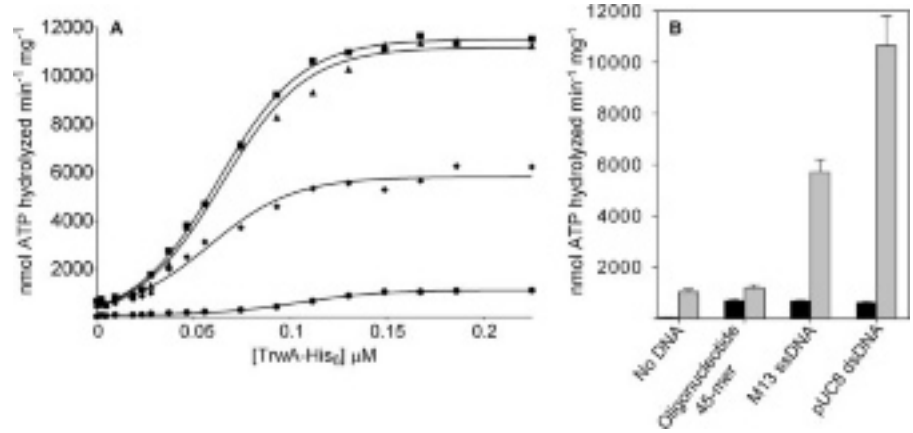

FIGURE 3. **Effect of TrwA-His$_6$ protein on TrwBΔN70-mediated ATP hydrolysis in the presence of different DNA substrates.** *A*, all reactions contained TrwBΔN70 (0.3 μM as monomer), 5 mM ATP, 52 μM base ssDNA, or 43 μM bp dsDNA and increasing concentrations of TrwA-His$_6$. Experiments were carried out with the coupled spectrophotometric assay, initiating the reaction by the addition of ATP. The effects of ssDNA (♦), supercoiled dsDNA (■), or linear dsDNA (▲) are shown. For comparison, activity in the absence of DNA has also been represented (●). *B* represents the TrwBΔN70 ATPase rates in the absence of TrwA-His$_6$ protein (in *black color*) or at saturating TrwA-His$_6$ protein concentrations (in *gray color*). Data are derived by averaging at least four experiments.

by linear or supercoiled dsDNA and 5–6-fold by M13 ssDNA, in the presence of saturating amounts of TrwA-His$_6$. Interestingly, the short 45-mer ssDNA oligonucleotide hardly increased ATPase rates above values obtained with TrwA-His$_6$ in the absence of DNA (Fig. 3, *panel B*).

The results obtained with relaxed and linear dsDNA were comparable to those obtained with supercoiled DNA. Moreover, there was no dependence on the sequence or length of the plasmid used. TrwA-His$_6$ was able to stimulate TrwBΔN70 ATPase activity independently of the presence or absence of its binding site on the *oriT* sequence (plasmid pSU1186 *versus* pUC8). The optimal TrwA/TrwB ratio was similar to that obtained in the absence of DNA.

The C-terminal Region of TrwA, but Not the N terminus, Stimulates TrwBΔN70 ATPase Activity—To obtain clearer insights into the functional aspect of the domain organization of TrwA and how different domains can affect TrwBΔN70 ATPase activity, we used truncated segments of TrwA containing the N-terminal region (residues 2–73) or the C-terminal region (residues 36–121). The ability of the truncated forms of the protein to bind *oriT* was previously analyzed, showing that TrwA-(2–73)-His$_6$ forms a stable DNA-binding domain, which binds specifically R388 *oriT*, whereas TrwA-(36–121)-His$_6$ does not bind DNA (8). The effect of each domain on TrwBΔN70 ATPase activity was analyzed by using the same assay as used with the full-length protein TrwA-His$_6$ (Fig. 4). Results show that TrwA-(2–73)-His$_6$ had no effect on TrwBΔN70 ATPase rates, neither in the absence nor in the presence of DNA (Fig. 4, *A* and *B*, respectively). On the other hand, hydrolysis of ATP was stimulated by TrwA-(36–121)-His$_6$, but a higher ratio TrwA/TrwB was required to obtain the same stimulatory values observed with the full-length protein. Therefore, we conclude that TrwA residues within the region 73–121 are involved in interactions with TrwB, being these interactions responsible for modulating TrwB ATPase activity.

Gel Filtration Chromatography Reveals a Stable Protein Complex between TrwB and the C-terminal Domain of TrwA—The ability of TrwA to interact with TrwB was further analyzed by gel filtration chromatography (Fig. 5*A*). TrwBΔN70 was incubated with TrwA-(36–121)-His$_6$, as described under "Materials and Methods." After 10 min at room temperature, samples were loaded into the column. Gel filtration chromatography showed the interaction between the two proteins, because they eluted in a single peak with an estimated mass of 68 kDa (*peak 3* in Fig. 5*A*). TrwA-(36–121)-His$_6$ protein eluted as a tetramer, with an estimated mass of 56 kDa (peak 2) and TrwBΔN70 eluted as a monomer, with an estimated mass of 51 kDa (peak 1). This result indicates that the complex

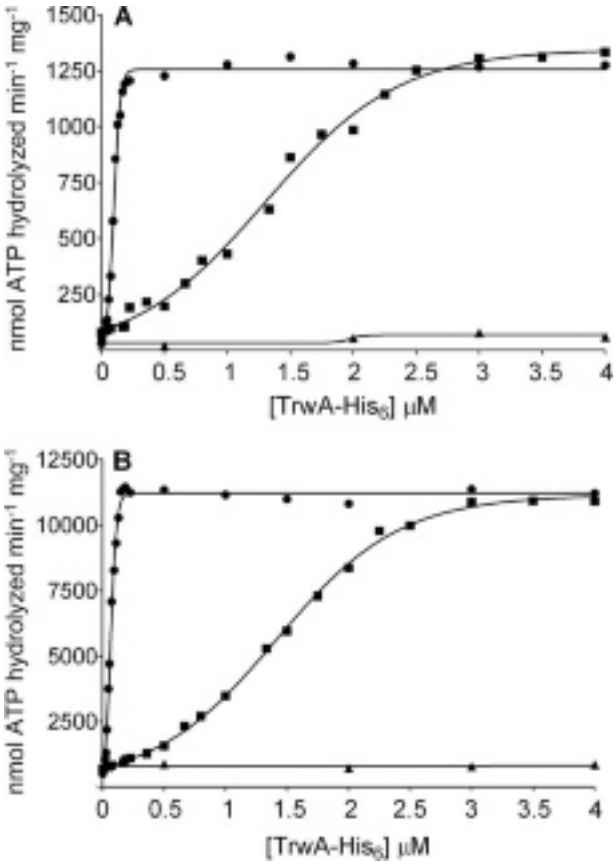

FIGURE 4. **Effect of the N-terminal and C-terminal domains of TrwA-His$_6$ on TrwBΔN70 ATPase rates.** Effect of two truncated domains of TrwA containing the N-terminal region (residues 2–73) or the C-terminal region (residues 36–121) on TrwBΔN70-mediated ATP hydrolysis in the absence (*A*) or presence of 43 μM bp DNA from plasmid pUC8 (*B*). Experiments were carried out with the coupled spectrophotometric assay. Reactions contained TrwBΔN70 (0.3 μM as monomer), 5 mM ATP and increasing concentrations of TrwA-His$_6$ (●), TrwA-(2–73)-His$_6$ (▲), or TrwA-(36–121)-His$_6$ (■).

between TrwB and the C-terminal domain of TrwA elutes with a 1:1 stoichiometry. The formation of the complex was confirmed by SDS-PAGE analysis of the resolved column fractions (Fig. 5*B*) and by cross-linking experiments (data not shown).

DNA and TrwA Promote TrwBΔN70 Oligomerization—Electron microscopic analysis of the samples (Fig. 6) revealed the presence of oligomeric TrwBΔN70 complexes when the protein was first incubated with either TrwA or the 45-mer oligonucleotide (oligonucleotide shown previously to promote the oligomerization and ATPase activity of TrwBΔN70) (18). The starting material for image analysis of TrwB-DNA complexes is shown in Fig. 6*A*. It corresponds to TrwBΔN70-DNA complexes obtained by gel filtration, in a peak that elutes with an estimated molecular of 440 kDa. The oligomeric TrwBΔN70 complex contains DNA, as shown by a correlated increase in the absorbance at 260 nm (18). After the alignment and classification of 2,463 of these particles, three class averages are shown in Fig. 6*B*. The size of these particles is approximately ~100 Å, which perfectly correlates to the size of hexameric TrwBΔN70 complexes solved by crystallography (15). The averaged electron microscopic images shown in Fig. 6*C* correspond to oligomeric TrwBΔN70 complexes obtained after incubating TrwBΔN70 and TrwA-His$_6$ proteins. In this case, samples were not previously chromatographed, since the formation of complexes in the presence of TrwA-His$_6$ is dependent on protein concentration. The size of these particles is similar to that of the TrwB-DNA complexes. In the absence of DNA or TrwA, TrwBΔN70 is in a monomeric state and no oligomeric assemblies could be visualized by electron microscopy (data not shown).

DISCUSSION

TrwB is a DNA transporter that also acts as a coupling protein during bacterial conjugation, recruiting the relaxosome to the inner membrane, where the protein is anchored. Previous biochemical and structural analysis revealed a DNA-dependent ATPase activity for TrwBΔN70 (18). However, the coupling mechanism between TrwB and the relaxosome remained unclear. Preliminary evidence for an interaction between TrwB and TrwA proteins was observed in co-elution experiments when TrwB was immobilized on an affinity column (11). The work presented here demonstrates that TrwA protein greatly stimulates the ATPase activity of TrwBΔN70. Specific interactions between both proteins allow the modulation of TrwBΔN70 ATPase activity by TrwA. As shown earlier (18), TrwB is an active ATPase in an oligomeric state, likely as a hexamer, and oligomerization is promoted by DNA binding. The results presented here suggest that TrwA also stimulates TrwBΔN70 ATPase activity by promoting TrwB oligomerization, since time course experiments show a lag phase that disappears when both proteins are preincubated together. This idea is also supported by electron microscopy analysis, which shows oligomeric TrwBΔN70 complexes when the protein is previously incubated with TrwA. At present, it is not possible to conclude if TrwA brings about the oligomerization of TrwB and then dissociates from the complex or whether it remains bound to the oligomer.

The major effect of TrwA protein was the enhancement of the effects of DNA on TrwB ATPase activity. Strikingly, in the presence of the TrwA protein, maximum values of ATPase activity were achieved with dsDNA substrates, which stimulated ATPase rates 10-fold. M13 ssDNA increased the activity by 5–6-fold, but the presence or absence of TrwA did not alter values exhibited with oligonucleotides (45- or 66-mer) (Fig. 3*B*). These results suggest that TrwBΔN70 works on DNA with high processivity only in the presence of TrwA.

The different results obtained with ssDNA and dsDNA led us to analyze TrwBΔN70 ATPase activity in the presence of different types of dsDNA substrates, and in the absence of TrwA. Another conclusion of this work is that TrwBΔN70 ATPase activity is stimulated by both ssDNA and dsDNA. As it happened in the presence of TrwA, both linear and circular supercoiled dsDNA stimulated TrwBΔN70 ATPase activity. Although the ATPase rates in the presence of dsDNA are high *in vitro*, the *in vivo* role for TrwB acting on dsDNA remains unclear, since it is generally accepted that ssDNA is the translocated substrate during conjugation (31, 32). This capability to work with both ssDNA and dsDNA has also been described for several hexameric helicases, such as DnaB or T7 phage gp4 helicase. These proteins unwind the DNA replication fork while encircling ssDNA, but are also able to

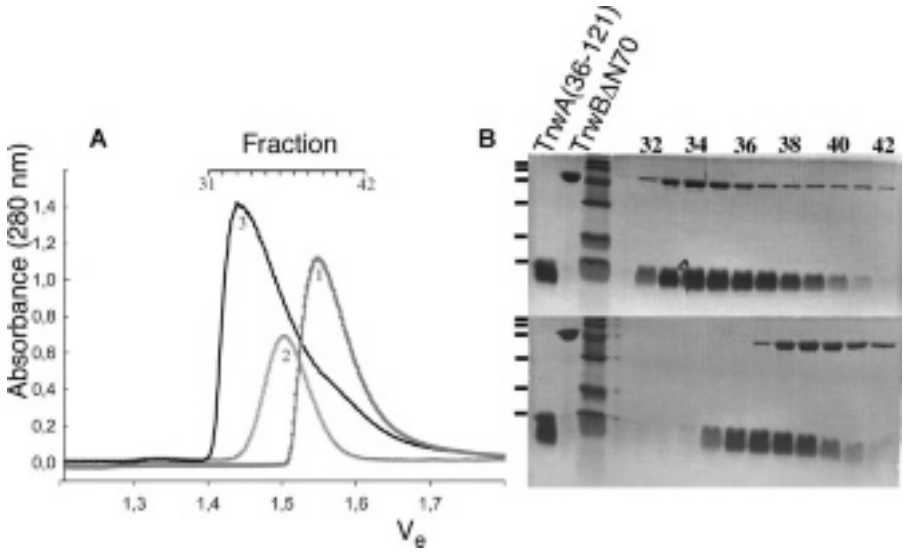

FIGURE 5. **Interactions between TrwBΔN70 and TrwA-(36–121)-His$_6$ determined by gel filtration chromatography.** *A*, protein samples were chromatographed separately, as described under "Materials and Methods." V_e represents the elution volume. Run 3 was obtained after incubation for 10 min of proteins TrwBΔN70 (208 μg) and TrwA-(36–121)-His$_6$ (343 μg). A single peak (*peak 3*) was obtained, with an estimated mass of 68 kDa. *Peaks 1* and *2* represent the separate elution profiles of TrwBΔN70 (208 μg) and TrwA-(36–121)-His$_6$ (343 μg), respectively. TrwA-(36–121)-His$_6$ elutes as a tetramer, with an estimated mass of 56 kDa. TrwBΔN70 elutes as a monomer, with an estimated mass of 51 kDa. *B*, SDS-PAGE of peak fractions. The first two lanes correspond to control lanes of TrwA-(36–121)-His$_6$ (900 ng) and TrwBΔN70 (600 ng). The third lane corresponds to molecular markers of 106, 77, 50.8, 35.6, 28.1, and 20.9 kDa. A 2.0-μl volume sample was taken from fractions 31–42 in each run. The gel on the *top* shows the fractions obtained from run 3. The gel at the *bottom* was obtained when 2.0-μl samples of equivalent fractions of runs 1 and 2 were mixed and then run together on a 15% SDS-polyacrylamide gel. Proteins were detected by Coomassie Blue staining.

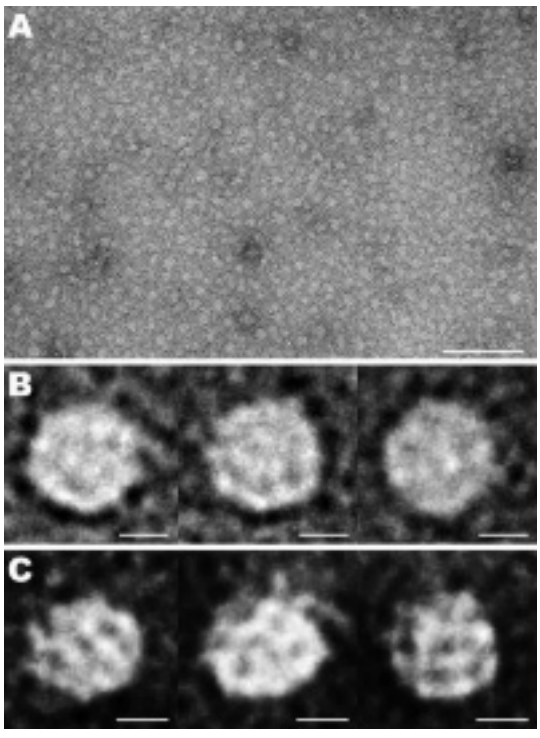

FIGURE 6. **Electron microscopy of TrwBΔN70-DNA and TrwBΔN70-TrwA complexes.** *A*, original images of TrwBΔN70-DNA complexes stained with uranyl acetate (scale bar, 100 nm). *B* and *C*, classes of averaged images (150–200 particles each) corresponding to TrwBΔN70-DNA and TrwBΔN70-TrwA complexes, respectively (*scale bars*, 5 nm).

actively translocate along a duplex, encircling both DNA strands (33). Other molecular motors, such as RuvB, involved in branch migration in the late stages of recombination, also share this capability (34). Therefore, this tracking capability on dsDNA might generalize to other DNA translocating motors, such as TrwB. Interestingly, both RuvB and T7 gp4 helicase work on DNA with a 5′ to 3′ polarity. Although in the case of TrwB, no helicase activity has been proven so far, DNA is also transferred in a 5′ to 3′ direction during bacterial conjugation. As discussed by Egelman (35), some proteins initially described as helicases, may use the energy of ATP hydrolysis to translocate along DNA or pump DNA without acting as classical unwinding enzymes.

How does TrwA modulate TrwB function as an ATP-dependent motor? Interactions between the two proteins occur *via* the C-terminal domain of TrwA, whereas its N-terminal domain has no effect on TrwBΔN70 ATPase activity. Therefore, the interacting region is located in TrwA between residues 73–121. This result is in agreement with experiments carried out with the F-homologue protein TraM. They define the C-terminal domain of the protein as the domain involved in interacting with the coupling protein (13).

To observe the interaction between the C-terminal domain of TrwA and TrwB directly, we analyzed the complex by gel filtration chromatography. The 1:1 stoichiometry of the complex reveals that the mixture of the two proteins causes the dissociation of TrwA-(36–121) tetramers to produce a monomeric state complexed with TrwB. The formation of the complex is dependent on protein concentration and, at present, we cannot conclude whether the 1:1 stoichiometry corresponds to a pre-loading complex or arises from dissociation of a complex of higher oligomeric state. The latter possibility is supported by the electron microscopic images obtained with the starting material, which show the existence of oligomeric TrwBΔN70 complexes.

Interactions between TrwA/TrwB proteins and the ability of TrwA to modulate TrwBΔN70 ATPase activity is similar to the mechanism used by other DNA-dependent molecular motors, such as the RuvA/RuvB system, to be targeted and assembled as hexamers on the DNA. RuvA is a tetrameric protein that binds DNA at the Holliday junction and facilitates the loading of RuvB protein onto DNA. RuvA enhances the ATPase activity of RuvB, a hexameric molecular motor required for branch migration during homologous recombination. The C-terminal domain of RuvA (domain III) inter-

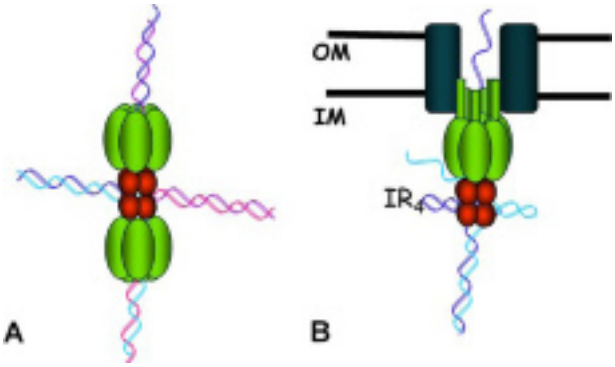

FIGURE 7. **A model for TrwB assembly on DNA mediated by TrwA.** *A*, RuvA protein (in *red*) is bound as a double tetramer to a square planar structure on the DNA, called Holliday junction. Each RuvA tetramer facilitates the assembly of one hexameric RuvB motor, represented in *green*. *B*, TrwA (in *red*) is represented also as a double tetramer bound to an inverted repeat called IR4, its specific recognition sequence at *oriT*, forming a hairpin in the figure. TrwB monomers anchored in the inner membrane are represented in *green*. *OM* and *IM* represent the outer and inner membrane, respectively. By analogy to the RuvA/RuvB paradigm, two TrwA tetramers have been drawn to maintain the structure on the DNA, but only one hexamer assembles, because it requires a ssDNA scaffold to oligomerize. In both cases, oligomerization and assembly of the hexameric motor on the DNA seems to be mediated by its interaction with TrwA or RuvA, tetrameric proteins, which previously bind specifically to the DNA. RuvB couples ATP hydrolysis to dsDNA pumping, whereas in bacterial conjugation ssDNA is the transported substrate.

acts with RuvB and modulates its ATPase activity. Furthermore, although the RuvA/RuvB motor works as a tetramer/hexamer complex, in gel filtration chromatography RuvA domain III and RuvB elute as a complex with a 1:1 stoichiometry (36). Such a complex was proposed as a preloading complex to recruit RuvB subunits and facilitate their assembly on the DNA. Thus, the RuvA/RuvB system exhibits strong mechanistic parallels with the results presented here for TrwA/TrwB. Some hexameric helicases, such as bovine papillomavirus replication initiator protein E1, also form a complex with the accessory protein E2 (37). Protein E2 determines the DNA binding specificity of the E1 hexamer at the origin of replication, forming a pre-replication complex. A model has also been proposed by which, following this association, the recruitment of additional E1 monomers would lead to the assembly of the E1 hexamer at the viral origin (30).

We can extrapolate this model to the TrwA/TrwB system to explain TrwA/TrwB interactions in the DNA translocation process toward the membrane (see Fig. 7). TrwA, as a tetramer bound to a specific inverted repeat in the *oriT* (IR4), would interact through the C-terminal domain with a TrwB monomer anchored in the inner membrane, promoting its oligomerization. The inverted repeat IR4, which is specifically recognized by TrwA, probably forms also a cruciform on the DNA. To summarize, the biochemical analysis presented here shows that TrwA, as RuvA, facilitates oligomerization and loading of TrwB on the DNA, modulating its activity. It is tempting to speculate that a universal mechanism to load hexameric motors onto DNA has been conserved in evolution. The model also explains the coupling role proposed for TrwB, connecting the relaxosome to the T4SS, in the membrane. Once the cleavage reaction is carried out by the relaxase TrwC, TrwB would couple the energy released from ATP hydrolysis to DNA pumping, as previously proposed (19).

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