TrwB is an integral membrane protein linking the relaxosome to the DNA transport apparatus in plasmid R388 conjugation. Native TrwB has been purified in monomeric and hexameric forms, in the presence of dodecylmaltoside from overexpressing bacterial cells. A truncated protein (TrwBΔN70) that lacked the transmembrane domain could be purified only in the monomeric form. Electron microscopy images revealed the hexameric structure and were in fact superimposable to the previously published atomic structure for TrwBΔN70. In addition, the electron micrographs showed an appendix, ~25 Å wide, corresponding to the transmembrane region of TrwB. TrwB was located in the bacterial inner membrane in agreement with its proposed coupling role. Purified TrwB hexamers and monomers bound tightly the fluorescent ATP analogue TNP-ATP. A mutant in the Walker A motif, TrwB-K136T, was equally purified and found to bind TNP-ATP with a similar affinity to that of the wild type. However, the TNP-ATP affinity of TrwBΔN70 was significantly reduced in comparison with the TrwB hexamers. Competition experiments in which ATP was used to displace TNP-ATP gave an estimate of ATP binding by TrwB (K_{ATP} = 0.48 mM for hexamers). The transmembrane domain appears to be involved in TrwB protein hexamerization and also influences its nucleotide binding properties.

Bacterial conjugation is a highly efficient and broad host range process during which DNA is transferred from a donor to a recipient bacterium across the envelope of both cells (for reviews, see Refs. 1 and 2). Conjugative DNA transfer requires three sets of plasmid-encoded proteins called Dtr (DNA transfer and replication), Mpf (mating pair formation) (3), and a coupling protein (2, 4). Dtr proteins process conjugative DNA through the formation of a nucleoprotein complex, the relaxosome, that cleaves and unwinds DNA in order to form the single DNA to be transferred (T-strand). The relaxosome “moves” to the transport site by means of the coupling protein, where the DNA transport apparatus (Mpf proteins) provides the pore for transport of the T-strand to the recipient cell (2, 4).

Plasmid R388 has the shortest known mobilization region (5, 6). Only three plasmid-encoded proteins, TrwA, TrwB, and TrwC, together with oriT, are involved in R388 mobilization (6). TrwC acts as a relaxase and a DNA helicase. It is responsible for both nick cleavage at oriT and T-strand unwinding (7, 8). TrwA is a small tetrameric protein, which binds two sites at oriT and enhances TrwC relaxase activity, while repressing transcription of the trwABC operon (9). The R388 relaxosome is composed of TrwA and TrwC bound to oriT DNA together with the host protein IHF. The latter inhibits TrwC nic cleavage by affecting the topology of the DNA site where TrwC has to act. By so doing, it is thought to modulate R388 conjugation (10).

The third plasmid-encoded protein necessary for R388 conjugative DNA processing is TrwB, which belongs to the coupling protein family. TrwB sequence analysis predicts an integral membrane protein of 507 residues (6, 11) and contains the characteristic NTP-binding motifs, reminiscent of those of the α and β subunits of F$_1$-ATPase (12, 13) and shared by the other coupling proteins (14). The transmembrane domain predicted by sequence analysis comprises the 70 N-proximal residues and includes two transmembrane helices and a small periplasmic domain in between. Although the coupling proteins appear to play an essential role in bacterial conjugation, few data are available on their biochemical mechanism. Protein TraD was the first coupling protein purified and subjected to biochemical analysis (15). Later, a soluble form of TrwB, lacking the N-terminal transmembrane segments and called TrwBΔN70, was purified (11) (see below). Recently, His-tagged TraG (RP4) and TraD (F) and truncated HP0524 (Helicobacter pylori) have been purified and partially characterized (16). Apparently, problems of aggregation and solubility precluded a more exhaustive characterization of these proteins. The poverty of results obtained in vitro underscores the difficulty in handling integral membrane proteins.

We observed that in the purification protocol, TrwBΔN70 behaved as a monomer. However, its crystal structure unveiled a molecule with six equivalent protein units (4). In this study, we have purified the integral membrane protein TrwB in its native form from an extract of TrwB-overproducing Escherichia coli cells. Unlike TrwBΔN70, TrwB appears in both monomeric and hexameric forms in solution. Since this behavior is different from the one corresponding to the TrwBΔN70 mutant, we infer that the transmembrane portion could play an important role in TrwB oligomerization. Our electron mi-
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**MATERIALS AND METHODS**

**Bacterial Strains and Plasmids—** _E. coli_ strains are described at the top of Table I, and plasmids are listed at the bottom. For conjugation experiments, the recA strains DH5α and UB1637 were used as donor and recipient strains, respectively.

**Oligonucleotides and Construction of Plasmids pBU1 and pBU2—** The oligonucleotides used in the present work were 1) GAAGGAGGATTCATATGTCAGCAG, which creates an NdeI site (underlined) and start codon at the N terminus of the _trwB_ gene, 2) GATTTCGGTGTCAGT, which creates a _KpnI_ site (underlined) at nucleotides 398–403 of the _trwB_ gene, and 3) GCAACAGCGCGTGACGTACC, which creates a _KpnI_ site (underlined) at nucleotides 404–409 of the _trwB_ gene and a K136T mutation in TrwB protein.

In order to produce TrwB protein, plasmid pBU1 was constructed as follows. Plasmid pSU4623, which carries the _trwB_ gene with a silent mutation to create a _KpnI_ site (11), was used as template for a PCR with oligonucleotides 1 and 2 (see above). The resulting 423-bp fragment was digested with _NdeI_ and _KpnI_ (fragment 1). Simultaneously, pSU4623 was digested with _KpnI_ and _BamHI_, the resulting 1.1-kb fragment was mixed with fragment 1, and this mixture was cloned in NdeI-BamHI-digested pSU4743 (pET-3a derivative) (20). This resulting construction contains TrwB protein under the control of the _T7_ promoter. Plasmid pBU1 was transformed to and stored in strain BL21 (DE3) and recipient strains, respectively.

For TrwB overproduction, plasmid pBU1 was introduced in _E. coli_ BL21 (DE3), a mutant strain of _E. coli_ harboring the plasmid pUB1 were used as donor and recipient strains, respectively. The resulting construction contains TrwB protein under the control of the _T7_ promoter. Plasmid pBU1 was transformed to and stored in strain UB1637 F′ (::DE3) (21). To produce TrwB-K136T, plasmid pBU2 was constructed as described before, and pSU4632 plasmid that carries the _trwB_ gene and a K136T mutation in TrwB protein.

**PROTEIN PURIFICATION**—TrwB was routinely purified from induced _E. coli_ BL21 C41 (DE3) cells. Cultures (6 g _E. coli_ for 15 min; the cells were then resuspended in the remaining volume of LB and frozen in liquid N₂. Frozen cells were thawed at 37 °C in 30 ml of buffer A (50 mM Tris-HCl (pH 7.8), 0.1 mM EDTA, 0.05% β-o-dodecylmaltoside) and applied at 1 ml/min to a 40 ml of cellulose phosphate P-11 (Whatman) column (2.5 × 8 cm) equilibrated with buffer B supplemented with 200 mM NaCl. Proteins were eluted in two steps: 500 mM NaCl in buffer B (fraction II, 100 ml) and 1 M NaCl in buffer B (fraction II, 50 ml). TrwB was eluted in both steps. This point on, fractions II and III followed separate, although parallel, purification protocols.

Fraction II was diluted to 150 mM NaCl by the addition of buffer B and loaded onto a 5-ml HiTrap-SP (Amersham Biosciences) column connected to an Amersham Biosciences FPLC system equilibrated with buffer B containing 150 mM NaCl. The column was washed with this buffer B until base line was reached. Proteins were then eluted from the column with a 150–1000 mM NaCl gradient in buffer B at a flow rate of 2.5 ml/min. TrwB, as a monomer, eluted at about 275 mM NaCl (Fraction III, 30 ml), and the hexameric form eluted at about 710 mM NaCl (Fraction III, 75 ml). Fraction III was concentrated by Centriprep and Centricon YM-50 systems (Amicon) to a final volume of 5 ml and loaded onto a 120-ml Superdex HR 200 column (16 × 80 cm) (Amersham Biosciences) by using Amersham Biosciences FPLC equipment. Gel filtration was performed in buffer B with 200 mM NaCl at a flow rate of 0.5 ml/min. The peak fractions corresponding to the monomer were pooled (fraction IV, 10 ml), and after glycerol was added to a 20% (v/v) final concentration, they were stored at −80 °C.

**Production of Antibodies—** Anti-TrwB protein monoclonal antibodies were prepared using the method described by Harlow and Lane (22). For antibody production, TrwB was purified by preparative SDS gel electrophoresis of a membrane fraction solubilized with Triton X-100 (1%, v/v) and applied first onto a DE52 (Whatman) column and then to a HiTrap Heparin (Amersham Biosciences) column. The band corre-

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1. The abbreviations used are: FPLC, fast protein liquid chromatography; TNP-ATP, 2′(or 3′)-O-(2,4,6-trinitrophenyl)adenosine 5′-triphosphate.
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RESULTS

**TrwB Purification**—The purification scheme of TrwB is shown in Fig. 1 and described under “Materials and Methods.” We have purified TrwB to apparent homogeneity from extracts of *Escherichia coli* BL21 C41 (DE3) carrying the trwB gene on a multicopy plasmid derived from pET3a. After induction with 1 mM β-β-thiogalactopyranoside, cells were grown at 25 °C to avoid formation of inclusion bodies and to promote integration of the protein into the cytoplasmic membrane. Membrane integration at this stage helped in obtaining functionally active protein.

Cells were harvested and membranes were obtained after breaking cells by sonication and subsequent centrifugation. After centrifugation, the following detergents were tested for TrwB solubilization: 1) 50 or 100 mM octyl glucoside; 2) 40 or 200 mM CHAPS; 3) 1% (w/v) Triton X-100; 4) 56 mM sodium cholate; 5) 1% (w/v) β-β-decylmaltoside. β-β-Dodecylmaltoside was the most effective in extracting TrwB from the membranes (data not shown). Furthermore, it was found that the addition of 600 mM NaCl improved protein solubilization.

Solubilized membranes were chromatographed on a cellulose phosphate P-11 column from which two fractions were obtained, at low (fraction II) and high (fraction IV) salt concentrations, respectively. These two fractions were separately treated from this stage onwards (see A and B, respectively, in Fig. 2). Fraction II (Fig. 2A, lane 2) was applied to a HiTrap-SP column and finally sieved on Superdex HR-200 where TrwB eluted to give 3.83 mg of homogeneous TrwB monomer (Fig. 2A, lane 4).

Fraction IV was applied to a HiTrap-SP column, and the resulting subfractions III-m and III-h were respectively applied to a Superdex HR-200 column, to give 0.5 mg of homogeneous TrwB monomer (Fig. 2B, lane 4) and to a Superose-6 column to give 0.34 mg of homogeneous TrwB hexamer (Fig. 2B, lane 6). The TrwB-K136T mutant protein was purified following the same purification protocol. However, the protein yield was strongly reduced in all fractions, and consequently only fraction II was obtained in significant amounts at the end of the purification. Thus, the studies involving the mutant protein were carried out with monomeric TrwB-K136T only. Note, however, that, although in small amounts, a fraction II’ of the mutant protein was obtained that, when processed through the HiTrap-SP column, could be resolved into monomers and hexamers (data not shown).

**Cellular Localization**—We examined the localization of TrwB by using specific antibody detection. *E. coli* D1210 cells containing the R388 plasmid were grown to exponential phase and lysed as described under “Materials and Methods.” Membrane fractions were prepared, and proteins were separated by SDS-PAGE. After immunoblotting, TrwB protein was specifically found in the inner membrane fractions of D1210 cells containing the R388 plasmid (Fig. 3). This result is fully compatible with the putative function of TrwB as a connector of the relaxosome (in the cytoplasm) to the transport system (in the membrane).

**Electron Microscopy of TrwB**—Samples of TrwB fraction IV-h were visualized by electron microscopy. Two preferred orientations of TrwB could be observed. The most abundant one can be defined as the side view (Fig. 4, upper panel A), and the processing of 860 of such particles generated an average image (Fig. 4, lower panel A) that was similar, albeit to a different resolution, to the lateral projection of the atomic structure of the TrwBΔN70 (Fig. 4, lower panel B), a mutant in which the first 70 residues, putatively involved in forming the membrane pore, have been deleted (30). Both projections
shared similar features, an orange-shaped structure with a channel traversing its central part. An important difference between both structures resides nevertheless in an \(25\-\text{Å}\)-wide appendix located at the bottom of the wild-type TrwB, which can be correlated with the transmembrane region of the protein and was not present in the structure of TrwB\(\text{N70}\). This region was not as well resolved as the major domain, and this may be due to the presence of detergent shielding its hydrophobic core. The wider amplitude of the bottom of the transmembrane domain (Fig. 4, lower panel A) may be due to its intrinsic flexibility, which generates a blurring of this region in the average image. The other preferred orientation of TrwB can be defined as the top view (Fig. 4, upper panel B). The average image of 530 of these particles (Fig. 4, lower panel C) revealed a structure similar to the projection of the atomic structure of the TrwB\(\text{N70}\) mutant along its 6-fold axis (Fig. 4, lower panel D). This average image presents, as its atomic counterpart, a central channel and six masses surrounding it, which undoubtedly
indicates that TrwB was purified as a hexamer.

**Nucleotide Binding to TrwB**—Upon protein binding, the fluorescence emission intensity of the fluorescent ATP analogue TNP-ATP (28) increases considerably; thus, it has been widely used to characterize ATP binding by a number of proteins (19, 31–36). As reported previously, TrwBΔN70 binds TNP-ATP as expected from the NTP binding signature in its amino acid sequence (11). Consequently, in this work, we used fluorescence spectroscopy to monitor the interaction of TNP-ATP with both TrwB and TrwB-K136T. The fluorescence emission spectrum of TNP-ATP in the presence of TrwB hexamers (Fig. 5) indicated a remarkable enhancement of the emission intensity and a blue shift (from 552 to 546 nm) of the wavelength of maximal emission. These were both indications that TNP-ATP had been transferred from the aqueous medium to the less polar environment inside the protein. Qualitatively similar spectra were obtained when monomeric TrwB was used (data not shown).

When TrwB was titrated with increasing amounts of TNP-ATP in the micromolar range, the relative increase in fluorescence became saturated, as seen in Fig. 6, for TrwB hexamers and monomers. This is the behavior expected for ATP-binding proteins (17, 18, 36–38). Our experimental results fitted well the saturation curves described by Equation 1, from which apparent dissociation constants for TNP-ATP ($K_{d(TNP-ATP)}$) could be computed. The corresponding values for TrwB hexamers and monomers were of 1.18 ± 0.38 and 2.35 ± 0.45 μM, respectively. These values should be compared with the $K_{d(TNP-ATP)} = 9.2$ μM observed for the truncated TrwBΔN70 (11).

The TNP-ATP binding has two components: specific binding to the ATP-binding site and nonspecific binding of the TNP moiety to nonpolar residues in the vicinity of the ATP-binding site (28). The nonspecific component can be eliminated by add-

**Fig. 3. Localization of TrwB protein.** Inner and outer membrane fractions were separated by sucrose gradient centrifugation as described under “Materials and Methods.” Samples of the different fractions were run on a 10% SDS-polyacrylamide gel that subsequently was subjected to immunoblotting with anti-TrwB IgGs. *E. coli* D1210 cells containing R388 plasmid. Lanes 2–5, cell extract, cytoplasmic, outer membrane, and inner membrane fractions, respectively. Lane 1, purified TrwB was used as control.

**Fig. 4. Upper panel,** gallery of negatively stained images of TrwB. A, gallery of side views. B, gallery of top views. **Lower panel,** two-dimensional negatively stained average images of the TrwB oligomer. A and C, average images of the side view and top view of TrwB, respectively. B and D, side and top projections, respectively, of the atomic structure of TrwBΔN70 mutant, to which the first 70 residues have been deleted (Protein Data Bank access code 1e9s) (30). All four images are shown at the same magnification. Bar, 50 Å.
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**FIG. 5.** Fluorescence spectra of TrwB-bound TNP-ATP in the presence and absence of ATP. Details of the experiment are described under “Materials and Methods.” Spectrum 1, spectrum of TrwB hexamers (1 μM); spectrum 2, TNP-ATP (8 μM); spectrum 3, TNP-ATP (8 μM) plus ATP (5 mM); spectrum 4, TrwB hexamers (1 μM) plus TNP-ATP (8 μM); spectrum 5, TrwB hexamers (1 μM) plus TNP-ATP (8 μM) plus ATP (5 mM).

**FIG. 6.** Fluorescence-monitored titration of TNP-ATP binding to TrwB protein. Successive aliquots of TNP-ATP stock solutions were added to a 0.4-ml sample of TrwB hexamers (1 μM) (▲) or TrwB monomers (0.8 μM) (●), and the fluorescence intensity (excitation 410 nm, emission 560 nm) was recorded after each addition. Each plotted value represents the difference in fluorescence intensity between the TrwB titration and the blank titration and was corrected as described under “Materials and Methods.” The lines represent the best fit to the data generated using Equation 1.

**FIG. 7.** Displacement of bound TNP-ATP by ATP in TrwB hexamers. Successive aliquots of ATP stock solutions were added to a solution containing TrwB hexamers (1 μM) (●) or TrwB monomers (0.8 μM) (○) and TNP-ATP (8 μM) in buffer A supplemented with 0.05% β-o-dodecylmaltoside and 20% glycerol. The fluorescence intensity (excitation 410 nm, emission 560 nm) was recorded after each addition. Each plotted value represents the difference in fluorescence intensity between the TrwB titration and the blank titration and was corrected as described under “Materials and Methods.” The solid line represents the best fit to the data generated using Equation 2.

Fluorescence intensity (a.u.)

Wavelength (nm)

Total TNP-ATP (μM)

0 1 2 3 4 5 6 7 8

ΔF/Fmax

ATP (mM)

0.00 0.05 0.10 0.15 0.20

0.25

protein was the first coupling protein that was purified in its native state and subjected to biochemical analysis (15). As a further step in the study of the coupling protein, we have overproduced and purified TrwB protein to near homogeneity. A remarkable aspect of the purification protocol is the fact that TrwB, but not TrwBΔN70, forms hexamers in solution indicating that the membrane domain plays a crucial role in the architecture of TrwB and also suggests a dynamic nature for the monomer-hexamer equilibrium.

Electron micrographs of TrwB (Fig. 4) support the data regarding the structure of nonligated TrwBΔN70 shown by Go-
mis-Rüth et al. (4, 30). The side view average image is similar, albeit to a different resolution, to the lateral projection of the atomic structure of TrwBAN70. Nevertheless, although both projections share similar features, at the bottom of the wild-type TrwB, an appendix of ~25 Å wide can be observed, which can be correlated with the transmembrane region of the protein, not present in the structure of TrwBAN70. The average top view reveals a structure similar to the projection of the atomic structure of TrwBAN70 along its 6-fold axis. This average image presents, as its atomic counterpart, a central channel and six masses surrounding it, indicating that native TrwB is purified as a hexamer.

TrwBAN70 has been also crystallized in the presence of nucleotides (4), showing that the nucleotide binding site is localized in the interface between monomers. The nucleotide binding experiments in this work provide TNP-ATP apparent dissociation constants somewhat smaller for the TrwB hexamers ($K_{d_{\text{TNP-ATP}}}$) = 1.18 μM) than for the monomers ($K_{d_{\text{TNP-ATP}}}$ = 2.35 μM). In a similar way, when bound TNP-ATP was displaced by ATP, we observed that the TrwB hexamers bound ATP with more affinity than the monomers (apparent dissociation constants 0.48 and 1.20 μM, respectively). Whereas the observed differences are significant, they could not support the idea of inactive half binding-sites that, upon hexamer formation, would complement each other to give fully active binding sites extending over two subunits. Rather, the independent subunits appear to possess active binding sites whose affinity for ATP is only partially increased by hexamerization.

The affinity of TrwBAN70 for TNP-ATP ($K_{d_{\text{TNP-ATP}}}$ = 9.20 μM) (11) is lower (by about 10-fold) than the one obtained for TrwB monomers. Therefore, removal of the transmembrane domain affects the nucleotide binding affinity to a higher extent than monomerization itself. This result suggests that the transmembrane domain also plays an important role in stabilization of the cytoplasmic soluble domain of TrwB.

The above results must be interpreted in the light of TrwB being an integral inner membrane protein. This is in agreement with the data from Panicker and Minkley (15) for the cellular localization of TraD (another coupling protein). A model for TrwB function has been proposed in a recent review (2). According to this model, TrwB acts in a second conjugation stage involving the cellular localization of TraD (another coupling protein). A model for TrwB function has been proposed in a recent review (2). According to this model, TrwB acts in a second conjugation stage involving the transport of the TrwC pilot protein and its trailing subunits.

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