TrwD, a Protein Encoded by the IncW Plasmid R388, Displays an ATP Hydrolysis Activity Essential for Bacterial Conjugation*

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A 1.7-kilobase pair segment from the conjugal transfer region of plasmid R388 DNA was cloned and sequenced. It contained trwD, a gene essential for plasmid R388 conjugation, for expression of the conjugal W-pilus and for sensitivity to phage PRD1. The deduced amino acid sequence of TrwD showed homology to the PulE/VirB11 superfamily of potential ATPases involved in various types of transport processes. A fusion of trwD with the glutathione S-transferase (GST) was constructed, and the resulting fusion protein was purified from overproducing bacteria. Factor Xa hydrolysis of GST-TrwD and further purification rendered TrwD protein with more than 95% purity. Antibodies raised against TrwD localized it both in the soluble fraction and in the outer membrane of Escherichia coli. TrwD is probably a peripheral outer membrane protein because it could be solubilized by increasing salt concentration to 0.5 M NaCl in the lysis buffer. Both purified GST-TrwD and TrwD could hydrolyze ATP. ATPase activity increased 2-fold in the presence of detergent-phospholipid mixed micelles. To study the importance of the nucleotide-binding site, Walker box A (GXXGXXGXXGK(T/S)), present in TrwD, the conserved lysine residue was replaced by glutamine. The mutant protein, expressed and purified under the same conditions as the wild type, did not exhibit ATPase activity. TrwD(K203Q) was not able to complement the mutation in trwD of the R388 mutant plasmid, suggesting the essentiality of the ATPase activity of the protein in the conjugative process. Furthermore, the dominant character of this mutation suggested that GST-TrwD(K432Q) was still able to interact either with itself or with other component(s) of the conjugative machinery. Bacterial conjugation is a DNA transport process that takes place with outstanding efficiency via a multienzymatic mechanism. The initial step in conjugation is the specific cleavage at oriT and the formation of a multiprotein complex called relaxosome. This step is fairly well understood at the molecular level. However, the events that lead the processed DNA from the donor to the recipient cell remain poorly understood (1, 2). More than ten proteins are required for this process. Several conjugation systems have been analyzed, and comparative studies of their respective gene sequences suggest a common molecular mechanism. Comparison among the individual proteins suggests that the PulE-like protein family is the one most widely spread among those implicated in the formation of the DNA transport complex (3). This family consists of proteins containing potential nucleotide-binding sites, and its members play essential roles in various processes of macromolecular transport in Gram-negative bacteria (4). Members of this family include proteins implicated in the export of various enzymes, exemplified by PulE, required for pullulanase secretion in Klebsiella oxytoca (5), and PilB, involved in pilus formation in Pseudomonas aeruginosa (3), as well as proteins implicated in DNA transport processes, like TrbB, essential in conjugative transfer of plasmid RP4 (6), ComG1, an essential competence gene in Bacillus subtilis transformation (7), and VirB11, required for transport of T-DNA from Agrobacterium tumefaciens into plant cells (8).

The common feature of the PulE-like proteins is a well conserved, centrally located region of about 100 amino acids that contains a nucleotide-binding motif (5). Its functional significance is not known, although it has been speculated that PulE, for example, might activate other proteins required for pullulanase secretion or else energize secretion via ATP hydrolysis (5). PulE, PilB, XcpR, and VirB11 have all been mutated in the Walker box A. pilB Walker box mutants do not assemble pili and xcpR Walker box mutants are blocked in toxin A and lipase secretion (9). pulE Walker box mutants are blocked in pullulanase secretion in a reconstituted system in Escherichia coli (10), and a mutant protein in Walker box for virB11 does not function in T-DNA transfer into plant cells (8). Thus, Walker box A is essential to the biological activity of this protein family. Although these observations suggest that these are nucleotide-binding proteins, attempts to demonstrate ATP binding or ATPase activity in members of this protein family (such as VirB11, XcpR, or PulE) have failed in the past (8–10). A recent study showed that VirB11 did not exhibit autophasorylation, ATPase, or ATP binding activity (8), contrary to a previous report (11). The gene trwD occurs in PILW (12). The amino acid sequence of TrwD indicates that it is a member of the PulE protein family, whereas various functional tests demonstrate that it is involved in the conjugal DNA transfer of plasmid R388 (12). As for the other members of its family, the predicted primary structure of TrwD includes the potential nucleotide-binding site formed by the Walker A and B consensus motifs. In this paper, we describe the DNA sequence of trwD and the purification and biochemical characterization of the ATPase activity of the protein.

EXPERIMENTAL PROCEDURES

Materials

Strains and Plasmids—Bacterial strains used were E. coli DH5a (F− Δ80dlacZΔM15 ΔlacZΔM15::Tn10lacZYA-argF)U169 endA1 hsdR17 relA1 gyrA96

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supE44 thi-1 recA1 deoR1 (13) and E. coli D1210 (recA lacI1079 pSLE1) (14). Plasmids used are listed in Table I.

### TABLE I

| Plasmid | Description | Reference |
|---------|-------------|-----------|
| pSU4058 | Ap’ P1L24, Rep (pMB8) | 15 |
| pSU4088 | Ap’ Km’ Rep (pMB8) pSU4058: TrnStacI | This work |
| pSU18 | Cm’ LacZa Rep (p15A) | 16 |
| pSU4116 | pSU18: trwD | This work |
| pGEX-3X | Ap’ lacI1 lacZa Rep (pMB8) gst | 17 |
| pSU4618 | pGEX-3X: trwD | This work |
| pGEX-3X: trwD (A1551C) | This work |
| pSU4039 | R388 (trwD: TrnStacI) | 15 |
| pSU4053 | Ap’ TRA1’ Rep (pMB8) | 15 |
| R388 | Tp’ Su’ TRA1’ IncW | 18 |

**TrwD Purification**—GST-TrwD fusion protein was purified from soluble extracts of D1210 (pSU4618), and purification was monitored by SDS-polyacrylamide gel electrophoresis and Coomassie Brilliant Blue staining or by Western blot analysis. 1 liter of culture in LB was grown at 37 °C to an A550 of 0.7. E. coli 111 mat was added, and cells were grown for an additional 2 h at 37 °C. All further manipulations were performed at 4 °C unless noted. Cells were harvested and resuspended in 50 mM Tris-HCl (pH 7.8), 1 mM EDTA, 0.5 mM NaCl, 3.3% sucrose (w/v). Cell lysis was carried out with 1 mg/ml lysozyme, 0.2% Triton X-100 (w/v), in the presence of 1 mM phenylmethylsulfon fluoride, 2 mM benzamidine, 1 mM β-mercaptoethanol, 2 mM β-mercaptoethanol, and 1 mM DTT. Inactive material was removed by centrifugation in a Type 70-1 Ti rotor (Beckman) at 45000 rpm at 4 °C for 30 min. Proteins in the supernatant (fraction I) were precipitated with 45% ammonium sulfate; the pellet was resuspended in buffer A (50 mM Tris-HCl (pH 7.8), 1 mM EDTA, 50 mM NaCl, 1 mM DTT) and dialyzed against the same buffer (fraction II). The dialyzed material was applied to an anion exchanger DE52 column (7 ml) equilibrated with buffer A. Proteins were eluted from the column by a two-step salt gradient (50−200 and 600−750 mM NaCl). GST-TrwD fusion protein was found in 650 mM NaCl (fraction III) and submitted to affinity chromatography in a 2-ml glutathione-Sepharose column (Pharmacia Biotech Inc.) equilibrated with phosphate-buffered saline buffer. GST-TrwD was eluted with 10 mM glutathione, 50 mM Tris-HCl (pH 7.8), 1 mM EDTA, 150 mM NaCl, 1 mM DTT. Protein-containing fractions were pooled and concentrated with Centricon 10 microcentrifugation filters, in buffer A (fraction IV).

The fusion protein was incubated with factor Xa (22) (50:1 w/v) in 20 mM Tris-HCl (pH 8.0), 100 mM NaCl, 2 mM CaCl2, for 8 h at 4 °C. The fragment corresponding to TrwD was eventually purified by a second affinity chromatography in a glutathione-Sepharose 4B column (the protein was found in the flow through). Fractions containing TrwD were concentrated with Centricon 10 microcentrifugation, in 50 mM Tris-HCl (pH 7.8), 1 mM EDTA, 150 mM NaCl, 1 mM DTT, 1 mM phenylmethylsulfon fluoride, 30% glyceral and stored at −70 °C where it remained stable for at least 6 months (fraction V).

To obtain further proof of the identity between the purified protein and the ATPase activity, 0.5 mg from fraction V protein were applied to a 1-ml fast protein liquid chromatography Mono Q HR 5/5 column (Pharmacia) equilibrated with buffer A. The column was eluted with a 20-ml gradient of this buffer of 0.05 to 1 M NaCl. 20 fractions were obtained and analyzed for the presence of TrwD. The ATPase activity of the relevant fractions was assayed by the spectrophotometric method (see below).

### Antibodies against TrwD and Immunological Techniques—GST-TrwD protein was boiled for 3 min in electrophoresis buffer and subjected to preparative scale SDS-polyacrylamide gel electrophoresis (23). A section of the gel containing the protein band (~500 μg of protein) was treated as indicated (24) and injected into New Zealand White rabbits to raise GST-TrwD-specific antisera, which jointly with horse-radish peroxidase-conjugated antirabbit immunoglobulin G was used to visualize TrwD protein by solid phase immunoassay.

Antibodies were purified by protein A-Sepharose CL CB (Pharmacia) chromatography following the manufacturer's recommendations. 5 μM GST-TrwD and 5 μM purified anti-GST-TrwD or preimmune antibodies were incubated for 2 h at room temperature. After incubation, mixtures were filtered through Microcon 100 filters to separate free protein from the immunecomplexes. ATPase activity was assessed in the filtrates by a spectrophotometric method (see below). In these assays, 100% ATPase activity corresponded to 0.32 μM ATP−min−1, and the figures shown represent the average of three independent experiments.

### Cell Fractionation—Cells were lysed by sonication, and the lysate was cleared by centrifugation. The pelleted membrane fraction was fractionated as described (25). The markers used were NADH oxidase for the inner membrane (26) and KDO for the outer membrane (27). The bands corresponding to TrwD in the immunoblot were quantified with a Dual Wavelength TLC Scanner CS-930 (Shimadzu).

### ATPase Assays—The formation of free phosphate from [γ-32P]ATP (3000 μCi/mm) was detected as described by Weinstein et al. (28). Reaction mixtures (40 μl) containing purified TrwD protein and [γ-32P]ATP were incubated in 50 mM Tris-HCl (pH 7.5), 5 mM MgCl2, 1 mM DTT, and nonradioactive ATP up to 5 μM unless indicated. Reactions were carried out at 30 °C for the specified times. Aliquots (1 μl from reaction mixtures were spotted onto polyethyleneimine-cellulose plates (Merck) and developed in 1 M formic acid, 0.5 M LiCl. ATP and Pβ spots were identified and quantified with a Molecular Imager GS-363 (Bio-Rad Laboratories).

Alternatively, a spectrophotometric method that included an ATP-regenerating system (29) allowed us to measure initial reaction rates.
ATPase Activity of TrwD

Fig. 1. DNA sequence of the EcoRI-HindIII fragment from pSU4116 plasmid (EMBL data base accession number X81123). PILW sequence, from nucleotide 8238 to 9949, is shown in capital letters, whereas sequences from other origins are in lowercase letters. The lower part of the sequence shows the overlap with the complementary strand of the previously described MOB W (mobilization region of the IncW plasmid EMBL accession number X81123).

The rate of ATP hydrolysis (dA/dt) was calculated as a function of time using a UVIKON 860 Kontron spectrophotometer. The absorption coefficients of GST-TrwD and TrwD were determined by comparing their absorption spectra under denaturing conditions.

Absorption Coefficient of GST-TrwD and TrwD—The absorption coefficient of GST-TrwD and TrwD was determined by comparing their absorption spectra under denaturing conditions versus native conditions, as described by Lohman et al. (30). The absorption coefficients of GST-TrwD and TrwD proteins in 6 M guanidinium hydrochloride were estimated to be $\varepsilon_{\text{max}} = 6.93 \times 10^4$ and $2.86 \times 10^4 \text{ M}^{-1} \text{cm}^{-1}$, respectively.

0.3 ml of reaction buffer (100 mM ethanolamine-HCl (pH 7.4); 5 mM MgCl$_2$, 0.6 mM EGTA, 100 mM KCl, 1 mM DTT, 0.16 mM NADH, 11.3 mM phosphoenolpyruvate, 0.01 mg/ml pyruvate kinase, 0.025 mg/ml lactate dehydrogenase) was added and incubated for 1 min at 37 °C. Finally, GST-TrwD (46.9 kDa) was incubated for 1 min at 37 °C. ATP (4 mM) was then added and incubated for 1 min at 37 °C. The absorption coefficient was determined from the Eadie-Scatchard plot, divided by $\varepsilon_{\text{max}}$.

Sequence Analysis of TrwD—A 9500-bp segment of DNA containing PILW in plasmid pSU4058 (15) was sequenced in both strands using Sequenase version 2. The complete sequence will be reported elsewhere (GenBank accession number X81123). 2

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2 S. Bolland, I. Sashe, M. Llosa, and F. de la Cruz, manuscript in preparation.

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\begin{enumerate}
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\end{enumerate}
Fig. 2. Conserved motifs in the PulE/VirB11 protein superfamily. Protein sequences were aligned using program PILEUP. Two one-aminos
acid gaps (shown by dots) near the His box were manually introduced to improve the alignment. The first block of sequences corresponds to
members of the PulE family (10), which showed p values ranging from 7 × 10−4 to 1 in a BLASTP search of TrwD. They were named according
to their SwissProt data bank names (GSPE_ERWCA, GspE from Erwinia carotovora; GSPE_ERWCH, GspE from Erwinia chrysanthemi; etc).

The alignment of the VirB11 family was used to build up a secondary structure prediction of the conserved segment according to the neural network algorithm of Rost and Sander (34). The second block corresponds to three potential open reading frames encoded by the completely sequenced archebacterium Methanococcus jannincum, which showed p values from 10−12 to 10−2 in the search.

Amino acids invariant within one of the families are shown in bold type. Asn-230 at the C-terminal end of GST is followed by Ser-2 of pGEX-3X so that a fusion protein of 587 amino acids is formed. Amino acids invariant within one of the families are shown in bold type.

![Diagram of ATPase Activity of TrwD](image)

**Table II**

Transfer frequencies of R388 and pSU4039 plasmids

| Species | rpsL | rpsM | pSU4039 | pSU4039 + pSU4618 | pSU4618 |
|---------|------|------|---------|-------------------|---------|
| E. coli | 6.1 × 10⁶ | <10 | 4.5 × 10⁶ | 6.0 × 10⁶ | 10 |
| R388 | 4.5 × 10⁶ | 10 | 6.0 × 10⁶ | 10 | 10 |
| R388 + pSU4618 | 4.5 × 10⁶ | 10 | 6.0 × 10⁶ | 10 | 10 |
| R388 + pSU4039 | 6.1 × 10⁶ | <10 | 4.5 × 10⁶ | 6.0 × 10⁶ | 10 |

Asn-230 at the C-terminal end of GST is followed by Ser-2 of TrwD. The TrwD protein obtained after cleavage with factor Xa rendered the TrwD moiety, which was purified to apparent homogeneity by means of a second affinity chromatography (Fig. 3). According to the method of Lohman et al. (30) (see "Methods" for details) five determinations at several protein concentrations yielded average absorption coefficients of ε⁰⁺ of 6.72 × 10⁵ and 2.58 × 10⁵ m⁻¹ cm⁻¹ for the native GST-TrwD and TrwD, respectively.

**Visualization and Localization of TrwD Protein**—Antibodies raised against the GST-TrwD fusion protein reacted against a 64-kDa protein present in total lysates from IPTG-induced D1210 (pSU4039) (Fig. 3). Furthermore, a 38-kDa reacting band was seen in extracts of D1210 (pSU4039) that contains the complete transfer region of R388. No immunoreactive material of this size was found in extracts from the trwD mutant D1210 (pSU4039), either induced or uninduced (Fig. 3). This result demonstrated that the antibody reacted specifically against protein TrwD and that TrwD protein was expressed by the wild type conjugative transfer region of plasmid R388.
ATPase Activity of TrwD

Fig. 3. Purification of TrwD. A, total proteins from crude lysates were electrophoresed through a SDS/12% polyacrylamide gel (I) and transferred onto nitrocellulose (II). The blot was probed with anti-GST-TrwD antiserum. Lane 1, induced E. coli D1210 (pSU4053); lane 2, induced (pSU4039), a trwD mutant; lane M, molecular mass markers. B, different steps in the purification of TrwD are shown by a SDS/10% polyacrylamide gel stained with Coomassie Blue (I) or by immunoblot (II). Soluble proteins from the IPTG-induced D1210 (pSU4618) (lane 1); eluted GST-TrwD from affinity column chromatography (lane 2); pooled and concentrated TrwD protein after a second affinity chromatography (lane 3) are shown (3 μg of protein in each lane). Molecular mass markers (lane M), with the size in kDa, are indicated at the right-hand side.

To ascertain the intracellular localization of TrwD, total lysates from IPTG-induced cultures of strains D1210 (pSU4053) and D1210 (pSU4058) (Table I) were separated into soluble, inner membrane, and outer membrane fractions, as shown under “Experimental Procedures.” The A280 pattern of the gradient fractions showed two major bands plus a faint, intermediate band. The upper and lower bands represented the cytoplasmic and outer membranes, respectively, as judged by the distribution of the outer membrane marker KDO and the cytoplasmic membrane marker NADH oxidase. As shown in Fig. 4, the lower band had an average KDO content of 14.4 mg liter\(^{-1}\) and an average NADH oxidase activity of 1.47 μmol ml\(^{-1}\) min\(^{-1}\). For the upper band these values were 2.1 mg liter\(^{-1}\) and 5.4 μmol ml\(^{-1}\) min\(^{-1}\), respectively. The intermediate band, which appeared between the two separated membranes, had also an intermediate content of both markers, suggesting that it probably consists of unseparated cell envelopes. Each subcellular fraction was found to have a unique protein profile when analyzed by SDS-polyacrylamide gel electrophoresis and stained with Coomassie Blue (data not shown), further suggesting an efficient fractionation. Proteins from an identical but unstained gel were transferred onto nitrocellulose, and the relative amount of TrwD protein present in subcellular fractions was determined by immunoblot analysis with anti-GST-TrwD antibodies (Fig. 4). TrwD protein was found in both the soluble and the outer membrane fractions.

TrwD ATPase Activity—ATP hydrolysis, both by GST-TrwD and TrwD protein, was initially detected with γ-\(^32\)P-labeled ATP. A radiochromatographic spot, corresponding to P\(_i\), was observed on thin layer chromatography of the enzyme-containing reaction mixtures (Fig. 5). A GTP-hydrolizing activity was also apparent, although the GTPase was ~30% of the ATPase activity. GST protein obtained from strain D1210 (pGEX-3X) did not exhibit any measurable ATPase activity (Fig. 5). TrwD ATPase activity was further characterized under ATP-regenerating reaction conditions (see “Experimental Procedures”). ATP hydrolysis was found to be proportional to protein concentration and linear with time (up to 90 min at 30 °C). The specific ATP hydrolase activity of TrwD, as measured by the spectrophotometric method, was of 4.5 nmol ATP/min • mg protein at 30 °C. As derived from the Eadie-Scatchard plot (data not shown), the apparent K\(_m\) for ATP was 0.5 mM, and the V\(_{\text{max}}\) value for a 3.5 μM TrwD concentration was 3.3 nmol ATP/min. To provide further proof that the purified ATPase activity corresponded to TrwD and not to a potential contaminating activity, a sample of the purified TrwD was subjected to an additional fast protein liquid chromatography MonoQ column purification that revealed only ATPase activities quantitatively correlated with TrwD protein abundance (data not shown).

To immunoprecipitate the ATPase activity of GST-TrwD, enough purified anti-GST-TrwD antibodies were added to precipitate more than 90% of the protein, as detailed under “Methods.” When the ATPase assay was carried out on the incubation mixtures, no differences in activity were observed among GST-TrwD, GST-TrwD with anti-GST-TrwD antibodies, and GST-TrwD with preimmune antibodies. This result suggested that even in the event that the immunocomplexes were formed, the interaction between antibody and protein did not significantly affect the active center of GST-TrwD. However, when incubation mixtures were ultracentrifuged in Microcon 100 filters, free protein was selectively filtered and consequently
separated from the antibody-sequestered TrwD. The ATPase activity of the free protein fraction was reduced to 21% after incubation with anti-GST-TrwD antibody and centrifugation, whereas 90% of the activity was retained after incubation with preimmune serum immunoglobulins and centrifugation.

Specific aspects of the ATPase activity were further characterized by the radiocromatographic method. Hydrolysis of ATP by TrwD required Mg\textsuperscript{2+}, whereas similar concentrations of Ca\textsuperscript{2+} reduced activity to 10%. As shown in Fig. 6, the optimum pH of the reaction was 9.0 (range 5.5–10). The effect of several agents over the ATPase activity was tested. The anionic detergent SDS had a clearly inhibitory effect on the ATPase activity (data not shown), whereas Triton X-100 could even stimulate the ATPase activity at concentrations above the critical micellar concentration (Fig. 7A) (the critical micellar concentration for Triton X-100 is 0.25 mM at 25 °C). Several phospholipid-detergent mixtures were also tested, but only for Triton X-100:PC:cardiolipin (3:1:1) micelles was a 2-fold increase in activity observed (Fig. 7B). Pure phospholipid vesicles did not have any measurable effect on the ATPase activity. Besides, the ATPase activity was not reduced by any of the ATPase inhibitors tested, N,N\textsuperscript{-}dicyclohexylcarbodiimide, thapsigargin, sodium orthovanadate, or oligomycin. No stimulating effect was observed in the presence of sodium sulfite (36).

Site-specific Mutagenesis of GST-TrwD—The predicted amino acid sequence of TrwD showed the sequence GKTGS\textsuperscript{K}K(T/S), which corresponds to the Walker box A, GXXGXXGK(T/S), a signature found in many ATP-binding proteins (Fig. 2). The highly conserved lysine in this motif is thought to interact with the γ-phosphate of ATP so that mutations in this position abolish ATP hydrolysis as well as greatly reducing nucleotide binding (Ref. 10 and references therein). To explore the importance of this potential nucleotide-binding site in the conjugative event, the gst-trwD gene was modified by oligonucleotide-directed mutagenesis. The conserved lysine in position 203 of TrwD (bp 8962 in Fig. 1) was changed to glutamine (K203Q). TrwD(K203Q) was overexpressed and could be purified using the same conditions as for the wild type protein. However, the mutant protein never showed a detectable ATPase activity.

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**Fig. 5.** ATPase activity of TrwD. a, GST from D1210 (pGEX-3X); b, wild type GST-TrwD from D1210 (pSU4618); c, mutant GST-TrwD from D1210 (pSU4631). A, radioactive method. Reactions were carried out for 1 h at 30 °C, as described under “Methods,” and the products were separated by thin layer chromatography on polyethyleneimine-cellulose plates after ATP hydrolysis. In all cases the protein concentration was 10 \( \mu \text{M} \), and the ATP concentration was 5 \( \mu \text{M} \). The arrow shows the direction of migration. B, spectrophotometric method. Reactions were carried out at 37 °C in the presence of an ATP-regenerating system (see “Methods”). Protein and ATP concentrations were 50 \( \mu \text{M} \) and 4 \( \mu \text{M} \), respectively. The times of addition of protein and ATP are marked by arrows.

**Fig. 6.** ATPase activity of TrwD versus pH. Reactions were carried out as described under “Methods” for 30 min at 30 °C. Protein and ATP concentration used were 10 and 5 \( \mu \text{M} \), respectively.

**Fig. 7.** ATPase activity of TrwD in the presence of the detergent Triton X-100 (A) and several phospholipids (B). ■, PC:Triton X-100 (1:3); ▲, PC:phosphatidylglycerol:Triton X-100 (1:1:3); ●, PC:cardiolipin:Triton X-100 (1:1:3). In both cases, reactions were carried out for 10 min as described under “Methods.” Protein and ATP were used at a concentration of 5 \( \mu \text{M} \).
(Fig. 5), despite the fact that the purification protocol was repeated more than six times during a 2-year period. Western blots, using an anti-GST-TrwD antibody, were used to check that the wild type and mutant proteins were expressed to similar levels, so that the differences shown should be attributed to a lack of enzymatic activity of the mutant protein. Total lysates either from D1210 (pSU4039 + pSU4618) and D1210 (R388 + pSU4618) cells expressing the native TrwD or from D1210 (pSU4039 + pSU4631) and D1210 (R388 + pSU4631) cells expressing the mutant protein were electrophoresed, transferred to nitrocellulose filters, and challenged with the anti-GST-TrwD antibody as shown in Fig. 3 for TrwD. The results showed indistinguishable levels of the wild type and mutant proteins both in exponential and in stationary phase cultures either in the presence of wild type R388 or in the presence of the mutant plasmid pSU4039 (data not shown). As to the in vivo function, TrwDK203Q could not be complemented to a mutation in trwD as was the wild type protein (Table II). This fact showed that the ATPase activity of TrwD is essential for conjugation. Furthermore, when the donor strain contained both R388 and the mutant pSU4631, R388 conjugation frequency decreased 1000-fold, underlying the dominant character of the mutation (Table II).

**DISCUSSION**

Bacterial conjugation involves the passage of a single-stranded DNA chain from a donor to a recipient bacterial cell. The molecular details of the mechanism of DNA transfer through membranes remain unknown despite active research (1). Plasmid R388 constitutes a good model system for the analysis of DNA transport because it appears to be genetically simpler than other conjugative plasmids (15).

The R388 DNA sequence responsible for conjugative pilus synthesis and export has been completely sequenced and contains 10 open reading frames responsible for the synthesis of 10 essential proteins in R388 conjugation and pilus formation (12). Of these, two proteins, TrwD and TrwK, contain potential NTP-binding motifs. As shown in Fig. 2, the range of proteins homologous to TrwD extend well outside the group of proteins involved in conjugation. Among the 10 proteins of plasmid R388 involved in pilus formation, TrwD is the one with the widest range of homologs, suggesting that it is playing a function that extends outside the conjugation “world.” Thus, it was considered of great interest to purify the protein and characterize its biochemical activities. Using a fusion vector system, we purified a GST-TrwD fusion protein from a soluble fraction of E. coli. In vivo GST-TrwD complemented a R388 trwD mutant to wild type transfer frequencies, indicating that it coded for a functional protein. The TrwD moiety was purified from the fusion protein.

Although the PulE family, to which TrwD belongs, contains conserved NTP-binding motifs (10), no clear evidence of such binding or NTPase activity has been produced to date. In particular, previous publications on similar proteins failed to show ATPase or ATP binding activities (4) and even claimed previous data that attributed a TrwD activity to VirB11 (8). However, bacteria expressing mutant PulE-like proteins in Walker box A, jointly with their complete transport systems, were inactive for transfer, suggesting the essentiality of the NTP-binding domain. We carried out four unrelated types of experiments to show that an ATPase activity is catalyzed by protein TrwD. (i) TrwD possesses an essential ATP hydrolyzing activity. The corresponding fraction obtained from the vector-only control strain possessed no detectable ATPase activity (Fig. 5). (ii) An additional, high resolution ion exchange chromatographic step showed a quantitative correlation between the ATPase activity of individual fractions and their TrwD protein content. (iii) Anti-GST-TrwD purified antibodies specifically sequestered the protein and the ATPase activity into nonfilterable complexes. (iv) When the conserved lysine of Walker box A was changed to glutamine, the mutant TrwD was no longer able to hydrolyze ATP (Fig. 5).

The absence of activity was accompanied by loss of in vivo functionality. The properties of the TrwDK203Q protein confirmed that the ATPase activity was essential to the role of TrwD in plasmid R388 conjugation. The dominant character of the mutation (Table II) suggests that the mutant protein still interacts with one or more other components of the conjugation machinery in a way that hinders their activity. Alternatively, if TrwD was oligomeric, a mutant protein still capable of oligomerization would produce an inactive oligomer resulting in a dominant-negative phenotype.

Why have previous workers failed to detect any ATPase activity in other members of the PulE-like family? The ATPase activity of TrwD is relatively weak when compared with the membrane-bound H+-ATPase or Na+-K+-ATPase of E. coli, and it may have been missed for this reason. The $K_m$ and $V_{max}$ values of TrwD were found to be similar to those previously reported for other well known ATPases in E. coli such as DnaK (37) and RecA (38). However, the ATPase activity profile of TrwD in the range of pH from 5.5 to 10.0 was different from that obtained for DnaK (37), which appeared to be a recalcitrant contaminating ATPase in VirB11 purification (8). Another possibility could be that some physiologically relevant inhibitor copurified with the TrwD-like proteins in earlier studies. Finally, it can be speculated that to activate their NTP-related activities, other members of the PulE family may need to interact with other protein(s) not present in overproducing bacteria that do not express complete systems. As shown in this work, such interactions, if existing, are not essential for TrwD to show a detectable ATPase activity.

The observation of TrwD as localized in both the soluble fraction and outer membrane (Fig. 4) may be indicative of a peripheral association of the protein to that structure. The inner bacterial membrane is almost certainly impermeable to the peptide, and the latter must cross the bilayer through some specific, unidirectional mechanism. Our results differ from the proposed localization of VirB11 (8) and PulE (4), which were both located in the inner membrane. Because pSU4053 contains about 20 plasmid copies per cell, the observation of a significant amount of protein in the soluble fraction may be the result of overproduction and thus of little physiological significance. However, preliminary results in our laboratories showing different electrophoretic mobilities between the soluble and membrane-bound fractions suggest that this distribution could be caused by a molecular difference between both forms of TrwD. This possibility remains to be explored further. The yield of TrwD purification increases significantly in the presence of 0.5 M NaCl. This is typical of peripheral or extrinsic membrane proteins and in our case indicates the release of the membrane-bound fraction. A related observation is the fact that ATPase activity is increased by pure detergent (at concentrations above critical micellar concentration values) or detergent-phospholipid micelles (Fig. 7) but not by phospholipids in bilayer form. This is suggestive of TrwD possessing hydrophobic patches on its surface, which would allow association to micelles or even a peripheral binding to bilayers (perhaps to intrinsic proteins in bilayers) but not insertion in the hydrophobic matrix of a membrane.

As for the role of TrwD in DNA transfer, at present we may only speculate. However, some of the data presented in this paper may be significant in shedding light on the putative role of TrwD. The combined observations that the protein (i)
membrane-associated, (ii) possesses an ATPase activity that is essential for conjugation, and (iii) shows higher ATPase activity in amphiphilic environments are strongly suggestive of an involvement of TrwD in some form of energy-dependent export mechanism. In addition, the fact that the Walker box mutation K203Q has a dominant character is compatible with TrwD making part of a supramolecular complex. Further studies on TrwD and other R388 gene products will be helpful in testing the above hypothesis.

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