JOURNAL OF BACTERIOLOGY, Oct. 1997, p. 6133–6137 Vol. 179, No. 19
0021-9193/97/$04.00+0

The Cytoplasmic DNA-Binding Protein TraM Binds to the Inner Membrane Protein TraD In Vitro

CLAUDIA DISQUÉ-KOCHEM AND BRIGITTE DREISEIKELMANN*

Mikrobiologie/Gentechnologie, Universität Bielefeld, Bielefeld, Germany

Received 27 December 1996/Accepted 14 July 1997

The cytoplasmic protein TraM is one of four essential gene products of the F factor which are involved in DNA transfer after mating pair formation. TraM binds to three specific sites within the *oriT* region. Besides regulation of its own synthesis, the precise function of TraM during conjugation is not yet known. In the present work, the affinity of TraM to TraD was studied in vitro by an overlay assay and by affinity chromatography. Whether the interaction between TraM and TraD causes a transient or permanent anchoring of the F factor to the site of transfer is discussed. A 35-kDa host membrane protein of yet unknown function also shows affinity to TraM and may be involved in this anchoring process as well.

Bacterial conjugation is a process during which DNA is transferred from a donor to a recipient across the envelope of both cells. One of the best-studied conjugative plasmids is the F factor of *Escherichia coli* (for review, see references 7, 8, 11, and 27). After pilus synthesis and mating pair formation during the conjugation of the F factor, four additional gene products of the *tra* operon are directly involved in a successful DNA translocation across the envelope of the donor. These products are encoded by the genes *traI*, *traY*, *traD*, and *traM*. Of these four essential Tra proteins, TraI is the best characterized. The TraI protein, also called helicase I, catalyzes the strand- and site-specific nicking of the F factor at *oriT* (16, 22). Furthermore, TraI unwinds duplex DNA in an ATP-hydrolysis-dependent reaction (1–3). The precise functions of TraY, TraD, and TraM are not yet known. It was recently shown in vivo that the nicking reaction of TraI is stimulated by TraY and the integration host factor (IHF) of *E. coli* (18). Both proteins bend DNA when bound to specific sites within the *oriT* region (14, 15, 17, 19, 26). Nelson and coworkers (18) propose that TraY and IHF form a nucleoprotein complex with *oriT* DNA which consequently can be recognized and nicked by TraI more efficiently.

While TraI and TraM function in the processing of the F DNA, TraD seems to be a component of the DNA transfer apparatus. It is generally accepted that export of the single-stranded F DNA across the envelope of the donor cell proceeds through a complex pore formed by or with participation of various Tra proteins. One of these proteins is probably TraD. The TraD protein with a molecular mass of about 82 kDa is located in the inner membrane (12, 20) and was shown to bind to DNA cellulose, indicating nonspecific binding to nucleic acids (20). The amino acid sequence deduced from the nucleotide sequence contains an ATP-binding motif (9) and preliminary data suggest a DNA-dependent ATPase activity of TraD (20).

TraM is a cytoplasmic protein with a molecular mass of 14.5 kDa. In the past, investigations of the TraM protein have mainly been focused on its regulatory function. Maximal *traM* transcription requires TraY in addition to expression of the *tra* operon. TraM binds to three sites within the *oriT* region of F (6). Two sites with high affinity for TraM (*sbmA* and *sbmB*) overlap with the two *traM* promoters, which means TraM autoregulates its own expression (21, 24). This stringent control leads to the synthesis of only about 25 to 30 molecules of TraM per cell (21). The third binding site with the lowest affinity for TraM (*sbmC*) as determined by DNase I footprinting is located between TraY (*sbyA*) and the IHF binding site (IHFB) close to *oriT*. Deletion of *smbC* shows the strongest effect on DNA transfer (9). Di Laurenzio and coworkers (6) suggest that the binding to *sbmC* may be crucial in the DNA transfer process because TraM could signal the cell that mating pair formation is completed and DNA transfer can be initiated. Conformational changes in the *oriT* region and interactions between TraI, TraY, TraM, and IHF at *oriT* are currently being investigated. If TraM signals the cell about the formation of stable mating pairs, it should at least temporarily contact the cell envelope. Previous studies of the localization of TraM support this idea, particularly since small amounts of the cytoplasmic protein could be found in the inner membrane (5, 6, 23).

In this study, we identify by different experimental approaches the TraD protein as one F gene product which mediates contact between the TraM protein and the inner membrane. We furthermore identified a new host membrane protein with a mass of 35 kDa which also shows affinity to TraM.

MATERIALS AND METHODS

Bacterial strains. The following *E. coli* strains were used in this study. *E. coli* C600 F$^-$ *thi-1 leuB6 lacY1 supE44 tonA21* was used for the expression of genes from pJF118EH (10) derivatives, and *E. coli* M15(pREP4) (Qiagen; Nal^r Str^r Rif^r *lac ara gal mtl* F$^-$) was used for the expression of genes from pQE40 (Qiagen). As an F$^+$ strain, we used *E. coli* CSH23 *supE44 rpsE* Δ(*lac-pro*) F′[*lac*$^+$ *proAB*$^+$].

Construction of plasmids for the overexpression of *traD*, *traM*, and *his-traM* genes. The *traD* gene was amplified by PCR with plasmid pRS31 (27) as the template with an upstream primer with the sequence 5′-CGGAATCATGAG TTTT-3′ and a downstream primer with the sequence 5′-CGGGATCCTCATC AGAAATCATCTCCCG-3′. The 2,150-bp amplification product was integrated as an *Eco*RI-*Bam*HI fragment into the expression vector pJF118EH. The hybrid plasmid was designated pCD150 and introduced into *E. coli* C600.

For cloning of the *traM* gene, a *Dra*I fragment from plasmid pRS30 (27) was integrated into the *Sma*I site of the expression vector pJF118EH. The hybrid plasmid was named pCD160.

The plasmid pCD165 was constructed as follows. By using a 5′ primer with the sequence 5′-GGATCCATGGCTAAGGTGAACCTG-3′ and a 3′ primer with the sequence 5′-AAGCTTTCTGATATTTGAACGAAGTC-3′, the *traM* gene was amplified by PCR with plasmid pRS30 (27) as a template. These primers introduce unique *Bam*HI and *Hin*dIII restriction sites at the 5′ and 3′ ends of the *traM*-amplified region, respectively. The amplification product was cloned into

* Corresponding author. Mailing address: Mikrobiologie/Gentechnologie, Universität Bielefeld, Postfach 100131, 33501 Bielefeld, Germany. Phone: 49-521-1065708. Fax: 49-521-1066015. E-mail: b.dreiseikelmann@biologie.uni-bielefeld.de.

pQE40 (Qiagen), creating a fusion between a sequence coding for six histines and the *traM* gene.

Overexpression of the cloned genes. One-liter cultures of *E. coli* cells were grown in TBY (10 g of tryptone, 5 g of yeast extract, 5 g of NaCl [per liter] [ph 7.5]) at 37°C to the logarithmic phase; isopropyl-β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 1 mM to achieve overexpression of the cloned genes. After 3 h of induction, the cells were harvested by centrifugation and stored at −70°C.

Purification of the TraD protein. Frozen cell paste of strain C600(pCD150) was resuspended in 10 ml of buffer A (50 mM Tris-HCl [pH 7.8], 10 mM EDTA). Lysozyme (4 mg) was added, and the suspension was incubated at 37°C until it became viscous. Enrichment of cell envelope proteins was performed as described previously (20) with the following modifications. Ammonium sulfate was added to 50% of saturation, and the solution was stirred on ice for 1 h and then centrifuged at 27,000 × *g* for 20 min. The lower layer was removed, and the pellet and the light yellow upper layer were dissolved in 2 ml of buffer D (50 mM sodium hydrogen phosphate [pH 7.8], 1% Triton X-100, 7 mM β-mercaptoethanol), dialyzed against the same buffer for 1.5 h, and stored at −20°C (fraction AS-TraD) if not used immediately.

For antibody production, the TraD protein was further purified with a phosphocellulose column and a Blue Sepharose column (fraction TraD).

Purification of TraM protein. Purification of the TraM protein from C600(pCD160) was done as described previously (5) with the following modifications. After ammonium sulfate precipitation (50% of saturation), the supernatant was dialyzed against 1.5 liters of TEBS buffer (50 mM Tris-HCl [pH 8.0], 0.1 mM EDTA, 50 mM NaCl, 7 mM β-mercaptoethanol) for 2 h and applied to a DEAE column (5-ml bed volume) equilibrated with TEBS. The column was washed with 10 bed volumes of TEBS buffer. Elution was performed with 60 ml of a linear NaCl gradient (50 to 1,000 mM) in TEBS buffer. The TraM protein eluted between 280 and 350 mM NaCl. These fractions were pooled, dialyzed against 2 liters of TEBS buffer for 2 h, and loaded onto a Blue Sepharose column (3-ml bed volume) previously equilibrated with the same buffer. After being washed with 10 volumes of TEBS buffer, adsorbed proteins were eluted with 35 ml of a linear 50 to 500 mM NaCl gradient in TEBS. Fractions eluting between 150 and 380 mM NaCl contained the TraM protein (fraction TraM).

Preparation of His-TraM protein for affinity chromatography. The frozen cell paste of an IPTG-induced *E. coli* M15(pREP4)(pCD165) culture (9 g from a 3-liter culture) was resuspended in 10 ml of buffer A (50 mM Tris-HCl [pH 7.8], 10 mM EDTA) with 4 mg of lysozyme and incubated at 37°C until the suspension became viscous. $MgCl_2$ (final concentration, 10 mM) and DNase I (80 μl of a 1-mg/ml solution) were added, and incubation was continued until the suspension was no longer viscous. The lysate was centrifuged at 27,000 × *g* for 20 min at 4°C. The supernatant was brought to 50% of saturation with ammonium sulfate, incubated on ice for 1 h, and centrifuged at 12,000 × *g* for 20 min at 4°C. The pellet was resuspended in 4 ml of buffer D (described above) with 300 mM NaCl and dialyzed against the same buffer for 1.5 h (fraction AS-His-TraM).

Affinity chromatography. An Ni-nitrilotriacetic acid (NTA) column (Qiagen; bed volume, 0.5 ml) was equilibrated with buffer D containing 300 mM NaCl, loaded with fraction His-TraM, and washed with 10 bed volumes of the equilibration buffer. The column was reequilibrated with buffer D without NaCl, loaded with fraction AS-TraD, and washed with 10 bed volumes of buffer D. Adsorbed proteins were eluted with a 7-ml gradient of 0 to 300 mM NaCl and 0 to 600 mM imidazole in buffer D.

Protein purification steps and fractions from the Ni-NTA column eluate were monitored by analyzing proteins on sodium dodecyl sulfate (SDS)–17.5 or 10% polyacrylamide gels (13).

Antiserum production. For antibody production, 100 μg of purified protein was separated in a preparative SDS-polyacrylamide gel. After electrophoresis, the protein was blotted onto a nitrocellulose membrane. Slices of nitrocellulose membrane containing the appropriate protein were cut out and used for immunization of rabbits (Eurogentec). Antisera were affinity purified by binding to membrane-bound antigen.

Western blot analysis (25). Total cell protein of *E. coli* or fractions from the Ni-NTA column were transferred to a nitrocellulose membrane (Optitran; Schleicher & Schuell) with a Trans-blot semidry transfer cell (Biometra). The transfer was performed as recommended by the supplier and allowed to proceed for 60 min. The membrane was blocked with 5% milk powder in 1× phosphate-buffered saline for 1 h at room temperature, followed by incubation with a suitable dilution (in the blocking buffer) of the first antibody against TraD and/or TraM for at least 1 h. Immunological detection was performed with alkaline phosphatase-coupled goat anti-rabbit immunoglobulin G (IgG) (Sigma; 1-h incubation) and 5-bromo-4-chloro-3-indolylphosphate toluidinium (BCIP) and nitroblue tetrazolium (NBT) (Gibco BRL) as the substrate according to the manufacturer's instructions.

Overlay assay (4). The overlay assay was carried out as described above for the Western blotting with the following additional steps. After the blocking, the membrane was incubated overnight with about 10 μg of purified TraM protein (fraction TraM), washed with the blocking buffer, and incubated with the first and the second antisera.

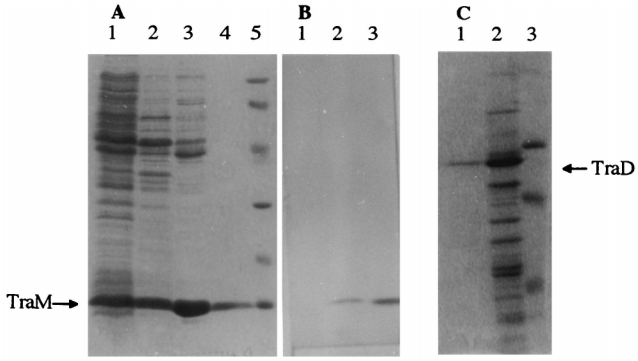

FIG. 1. Purification of TraM and TraD and specificity of the anti-TraM antiserum. (A) Purification of TraM, as shown with a Coomassie-stained SDS–17.5% polyacrylamide gel. Lanes: 1, crude extract of induced *E. coli* C600(pCD160); 2, supernatant after ammonium sulfate precipitation; 3, pooled fractions eluted from DEAE; 4, pooled fractions eluted from Blue Sepharose; 5, protein standards (phosphorylase *b*, 94 kDa; bovine serum albumin, 67 kDa; ovalbumin, 43 kDa; carbonic anhydrase, 30 kDa; soybean trypsin inhibitor, 20.1 kDa; α-lactalbumin, 14.4 kDa). (B) Western blotting with anti-TraM antiserum. Lanes (total protein of): 1, C600; 2, CSH23; 3, C600(pCD150) (IPTG induced). (C) Coomassie-stained SDS–10% polyacrylamide gel of purified TraD. Lanes: 1, pooled fractions from Blue Sepharose; 2, crude membrane proteins from induced *E. coli* C600(pCD150); 3, protein standards (94, 67, and 43 kDa [see Fig. 4]).

RESULTS

Purification of the proteins TraM and TraD. The *traM* and *traD* genes were cloned into the expression vector pJF118EH. *E. coli* C600 cells carrying the recombinant plasmid pCD150 (pJF-*traD*) or pCD160 (pJF-*traM*) were induced with IPTG for 3 h. The TraM and TraD proteins were isolated from these induced cells as described in Materials and Methods. All purification steps were analyzed by SDS-polyacrylamide gel electrophoresis (PAGE) or by binding to *oriT* DNA for purification of TraM only. Coomassie-stained gels of purified TraD and TraM protein are shown in Fig. 1A and C. The specificity of the affinity-purified polyclonal anti-TraM antiserum is demonstrated in Fig. 1B.

Analysis of proteins with affinity to TraM by an overlay assay. Since we were interested in the identification of partners interacting with TraM, the method of choice was the overlay assay. Theoretically, TraM could interact with either host proteins and/or F-encoded Tra proteins. If TraM should exhibit a signalling function in the cell, the interaction partners may be membrane proteins. Therefore, TraD seemed to be a promising candidate for a systematic analysis. For reasons outlined above, overlay assays were performed with total protein extracts from the F^- strain C600 and the F^+ strain CSH23 with and without pCD150 (pJF-*traD*). Proteins were separated by SDS-PAGE and transferred to a nitrocellulose membrane. After blocking of free binding sites and partial renaturation of proteins, the membrane was incubated with purified TraM protein (fraction TraM). This incubation allows TraM to bind to its potential partners, which are immobilized on the nitrocellulose. Subsequently the membrane was incubated with the primary antibody anti-TraM and with the alkaline phosphatase-conjugated secondary antibody anti-rabbit IgG. The membranes were treated with the substrates BCIP and NBT to detect signals at positions where TraM is bound to another protein. Figure 2B shows the result of the overlay assay. A signal corresponding to a protein with a molecular mass of 35 kDa can be detected in all three lanes; a second signal which is only present in the F^+ TraD-overproducing strain is found at the position of the TraD protein (82 kDa). Identical signals

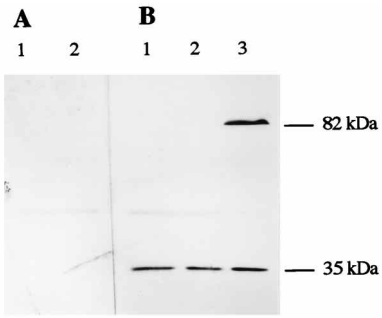

FIG. 2. Overlay assay of total protein with TraM. Samples of total proteins were separated in an SDS–17.5% polyacrylamide gel, blotted onto nitrocellulose, and incubated subsequently with TraM, anti-TraM antiserum, anti-rabbit IgG antiserum coupled with alkaline phosphatase, and the substrates NBT and BCIP. (A) Control without TraM incubation. Lanes: 1, CSH23; 2, CSH23(pCD150) induced. (B) Incubation with TraM. Lanes: 1, C600; 2, CSH23; 3, CSH23 (pCD150) induced.

were obtained with extracts of TraD-overproducing F^- strain C600 (data not shown). As a control, a second nitrocellulose membrane with total protein of CSH23 and CSH23(pCD150) was not incubated with TraM and showed no signals, as expected (Fig. 2A). Thus, TraM interacts with a 35-kDa host protein and probably also with the TraD encoded by pCD150. The lack of the 82-kDa signal in lane 2 (Fig. 2B) can be explained by the small amounts of TraD in F^+ cells and the poor transfer of the protein from the SDS gel onto the membrane. However, we were able to detect a very faint signal in similar experiments with total protein of F^+ cells.

To prove the assumption that the signal detected in total protein of strain CSH23(pCD150) is indeed caused by interaction with the inner membrane protein TraD, crude membrane protein fractions (fraction AS-TraD) of this strain and purified TraD protein were transferred to a nitrocellulose membrane and analyzed by the overlay technique. A signal corresponding to a protein with the expected molecular mass of 82 kDa was observed in both samples (Fig. 3, lanes 1 and 2), indicating that indeed TraD is the protein interacting with TraM. In addition, the 35-kDa host protein can also be detected in the membrane protein fraction (Fig. 3, lane 1). This indicates that the cytoplasmic TraM protein may contact the inner membrane by association with the F-encoded TraD protein and a second host-encoded membrane protein of yet unknown function.

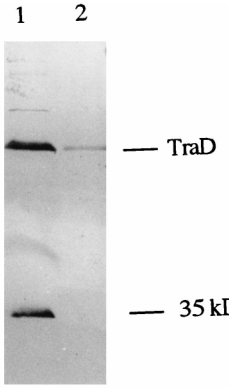

FIG. 3. Overlay assay of blotted membrane proteins with TraM. Proteins from crude membrane preparations of induced CSH23(pCD150) cells (fraction AS-TraD) (lane 1) and purified TraD (fraction TraD) (lane 2) were separated by SDS-PAGE (10% polyacrylamide). The overlay assay was performed as described in the legend to Fig. 2.

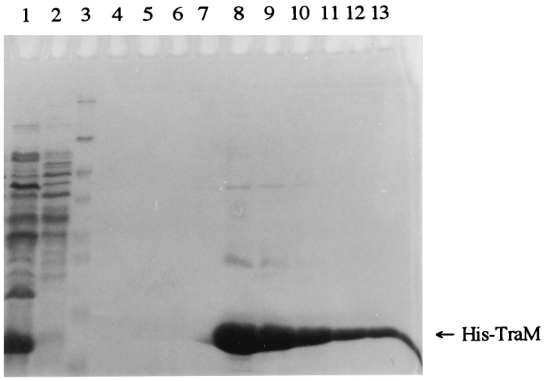

FIG. 4. Affinity chromatography of His-TraM on an Ni-NTA column. Lanes represent results from an SDS–17.5% polyacrylamide gel with the following samples: 1, total protein of induced *E. coli* M15(pREP4)(pCD165); 2, flowthrough of the column; 3, protein standards (myosin, 250 kDa; bovine serum albumin, 98 kDa; glutamic dehydrogenase, 64 kDa; alcohol dehydrogenase, 50 kDa; carbonic anhydrase, 36 kDa; myoglobin, 30 kDa; lysozyme, 16 kDa; aprotinin, 6 kDa; insulin B chain, 4 kDa); 4 to 13, proteins eluted from the column with an imidazole gradient.

Isolation of a TraD-TraM complex by affinity chromatography. The overlay technique has the disadvantage that the immobilized protein is denatured during the SDS-PAGE. To overcome this problem, we performed affinity chromatography between TraM protein bound to a ligand by a His tag at the N terminus and TraD protein, both in their native conformation.

In order to create the tagged protein, the *traM* gene was amplified by PCR and integrated into the expression vector pQE40 (Qiagen) in frame with six codons for histidine residues encoded by the vector. The His-tagged protein (His-TraM) produced from pCD165 strongly binds to an Ni-NTA resin. Any other host proteins that bind nonspecifically to the NTA resin can easily be removed under stringent conditions, without affecting the binding of the His-tagged protein. As shown in Fig. 4, the His-TraM protein can be eluted with an imidazole gradient in buffer D.

For immobilization, the His-TraM protein (fraction AS-His-TraM) of *E. coli* M15(pREP4)(pCD165 cells) was loaded onto an Ni-NTA column until the column was saturated with His-TraM protein. In order to avoid unspecific binding of other proteins to the column, all binding sites must be occupied by the His-TraM protein. After extensive washing, a fraction of crude membrane proteins (fraction AS-TraD) of induced *E. coli* C600(pCD150)(pJF-*traD*) was applied to the column, and the column was washed again. If the TraD protein is able to interact with TraM, it should bind to the immobilized His-TraM, and the complex can be eluted with an imidazole-NaCl gradient. Previous overlay assays with His-TraM confirmed that the His tag does not prevent the interaction with TraD (data not shown). Figure 5A shows SDS-PAGE with the flowthrough of two Ni-NTA columns, one saturated with His-TraM and the second saturated with another His-tagged protein, after being loaded with membrane protein fractions containing TraD. TraD is found in the flowthrough of the TraM-free column, but it is missing in the flowthrough fraction of the His-TraM-saturated column (compare lane 5 with lane 4 in Fig. 5A). From the TraM column, TraD is coeluted with TraM in an imidazole-NaCl gradient (Fig. 5B, lanes 1 to 9). Thus, the proteins TraM and TraD interact with each other in vitro.

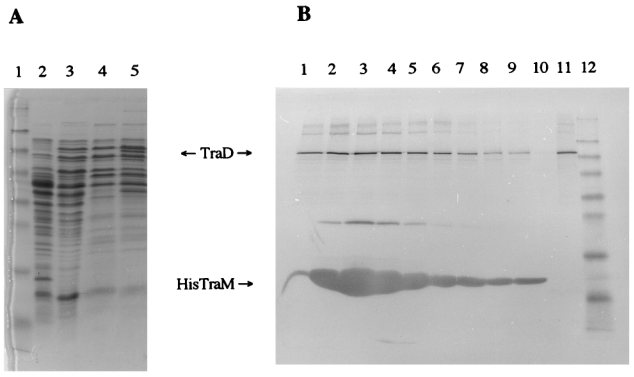

FIG. 5. Coelution of TraD and His-TraM from an Ni-NTA column. After saturation of the column with His-TraM (fraction AS-His-TraM), proteins from crude membrane preparations of induced C600(pCD150) cells (fraction AS-TraD) were applied to the column. Elution of bound proteins was done with an imidazole-NaCl gradient. Fractions were separated in an SDS–17.5% polyacrylamide gel. (A) Coomassie-stained SDS-polyacrylamide gel. Lanes: 1, protein standard (see Fig. 4); 2, total protein of C600(pCD150) uninduced; 3, C600 (pCD150) induced; 4, flowthrough from a column saturated with His-TraM; 5, flowthrough from a column not saturated with His-TraM. (B) Western blot analysis with anti-TraM and anti-TraD antisera. Lanes: 1 to 9, samples of proteins eluted with an imidazole-NaCl gradient; 10, total protein of C600; 11, total protein of C600(pCD150) induced; 12, protein standard (see Fig. 4).

DISCUSSION

In the present study, we identified the TraD protein as being involved in changing the intracellular localization of TraM. TraM is a soluble protein without membrane-spanning hydrophobic regions which binds to specific sites of the F DNA in the region of *oriT*. Di Laurenzio et al. (6) observed a small amount of TraM in the inner membrane fraction. Here we demonstrate through biochemical experiments that TraM binds to the inner membrane protein TraD and to a second host-encoded membrane protein with a molecular mass of 35 kDa of yet unknown function.

Different consequences from binding of TraM to F DNA and TraD are conceivable. TraD plays an essential role in DNA transfer and is thought to be a component of the transfer apparatus. Therefore, binding of TraM to TraD may physically anchor the F DNA to this transfer apparatus. Because the DNA-binding sites of TraM are located in the *oriT* region, the sites of DNA processing could be positioned to the site of transfer in the way that the nicked and unwound single strand can directly pass the membrane. Additionally, the binding sites of TraM are transferred at last to the recipient, meaning that the physical connection between the DNA and the pore would persist during the whole transfer process. The function of a membrane anchor implicates an at least temporary binding of TraM to TraD and at the same time to F DNA.

However, these in vitro experiments show that binding of F DNA to TraM is not a prerequisite for the binding to TraD. That TraM could signal the cell that mating pair formation is completed and DNA transfer can start has been discussed previously. However, until now, which way TraM is involved in the signal transduction has not been known. One explanation may be that formation of the complete and functional transfer apparatus results in modification and activation of TraM protein. The activated form of TraM may be released from the site of mating pair formation and successively bind to *oriT*. Whereas the inactive form of TraM may bind to DNA without effecting the DNA processing reaction, the activated form of TraM could initiate this reaction. A modification of TraM has not yet been detected. Purified TraM protein binds to *oriT* DNA and to TraD with similar affinities; thus, there is no indication for different forms of the protein. Nevertheless, a modification such as phosphorylation by the hypothetical ATPase of TraD or a change of the polymerization grade cannot be excluded.

Taking together the hypotheses as discussed above, we conclude that the F factor may be anchored to the transfer apparatus via TraM-TraD association. This may not only facilitate the DNA transfer by guiding the DNA close to the site of transfer, but may also position the proteins involved in processing into the neighborhood of the transfer apparatus. Mating pair formation may cause a cascade of conformational changes or modifications of proteins which starts at the outside of the cell and is transferred across the cell envelope to the proteins functioning in processing. TraD and TraM may be targets for these modifications. Consequently, a nucleoprotein complex ready for processing and transfer would be formed.

ACKNOWLEDGMENTS

This work was supported by a grant from the Deutsche Forschungsgemeinschaft (Dr 1894-1).

We thank S. K. Hemschemeier for critical reading of the manuscript.

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