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TrbK, a Small Cytoplasmic Membrane Lipoprotein, Functions in Entry Exclusion of the IncPα Plasmid RP4

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TrbK is the only plasmid-encoded gene product involved in entry exclusion of the broad-host-range plasmid RP4. The corresponding gene, *trbK*, coding for a protein of 69 amino acid residues maps in the Tra2 region within the mating pair formation genes. TrbK carries a lipid moiety at the N-terminal cysteine of the mature 47-residue polypeptide. The mutant protein TrbKC23G cannot be modified or proteolytically processed but still acts in entry exclusion with reduced efficiency. An 8-amino-acid truncation at the C terminus of TrbK results in a complete loss of the entry exclusion activity but still allows the protein to be processed. TrbK localizes predominately to the cytoplasmic membrane. Its function depends on presence in the recipient cell but not in the donor cell. TrbK excludes plasmids of homologous systems of the P complex; it is inert towards the IncI system. The likely target for TrbK action is the mating pair formation system, because DNA or any of the components of the relaxosome were excluded as possible targets.

Soon after the discovery of the transfer of genetic material by bacterial conjugation, Lederberg and coworkers found that the presence of a plasmid in the recipient cell inhibits transfer of an identical or a closely related plasmid (38). Two distinct processes were found to be responsible for this phenomenon, incompatibility and entry or surface exclusion (48). Entry exclusion functions of several self-transmissible plasmids and of a mobilizable plasmid have been described. These include the plasmids of the incompatibility groups F (1, 2), IncI (20, 24, 25, 27, 56), IncP (22, 42, 44), and IncN (55, 75, 76) and the mobilizable plasmid ColE1 (77), and the sex pheromone plasmids pAD1 and pCF10 of gram-positive bacteria (18, 35, 49, 73).

In general, entry exclusion functions interact only within homologous transfer systems. Entry exclusion (Eex) systems acting on related transfer systems belong to one entry exclusion group. The entry exclusion systems of the F and F-like plasmids are the most intensively studied (65, 74). Two genes encoded by the F Tra operon, *traT* and *traS*, were shown to contribute independently to the Eex phenotype (2, 33). The exclusion functions of $IncI_1$ plasmids R144, R64, and ColIb were shown to belong to one entry exclusion group (26). Two polypeptides responsible for entry exclusion were identified for R144 and R64 (20, 27). Other transfer systems like that of the IncN plasmid pKM101 encode only one gene product which functions in entry exclusion (55).

Although the entry exclusion determinants of several plasmids are known, little information is available about the mechanisms by which they inhibit the conjugative DNA transfer into the recipient cell. TraT of the F plasmid is a surface-exposed outer membrane lipoprotein and was thought to inhibit stable mating pair formation (1, 54, 65). TraS of the F plasmid and ExcA, the 19-kDa polypeptide of the $IncI_1$ plasmid R144, were shown to localize to the cytoplasmic membrane and were thought to inhibit DNA transfer after mating pairs have been established (3, 25, 28, 65).

To further understand the entry exclusion process, we studied the structure and function of the entry exclusion protein of the broad-host-range IncPα plasmid RP4 (52). It has been previously shown that a single gene, *trbK*, is responsible for the Eex phenotype of RP4 (22). The gene is located within the transfer operon Tra2, encoding the RP4 mating pair formation (Mpf) system (40, 41). It is nonessential for RP4-specific transfer and mobilization of the IncQ non-self-transmissible plasmid RSF1010 (22). *trbK* encodes a small hydrophilic polypeptide of 69 amino acid residues, which contains a lipoprotein signature (40). Here we describe functional and structural studies of TrbK and its localization within the cell envelope. Possible mechanisms of its action in entry exclusion are discussed.

MATERIALS AND METHODS

Strains, phages, and plasmids. *Escherichia coli* K-12 strains used in this study were SCS1 [*recA1 endA1 gyrA96 thi-1 hsdR17* (r_K^- m_K^+) *supE44 relA1*], a DH1 derivative (23) used as the host for plasmids; HB101 (*hsdS20 recA13 ara-14 proA2 lacY1 galK2 rpsL20 xyl-5 mtl-1 supE44*) (7); W3110T$^-$ (*thy deoC*) (5), the donor strain for entry exclusion experiments; and XL-1 Blue (*endA1 hsdR17* (r_K^- m_K^-) *supE44 thi-1 recA1 gyrA96 relA1* Δ(*lac*) [F′ *proAB lacI*^{q}ZΔM15 Tn*10* (Tc^r)]) (9), the host for M13mp18/19 derivatives. The naturally restriction-deficient *E. coli* C strain W2438 (12) was used as the recipient in entry exclusion experiments. Cells were grown in either yeast tryptone medium (47) buffered with 25 mM 3-(*N*-morpholino)-propanesulfonic acid (sodium salt, pH 8.0) and supplemented with 0.1% glucose and 25 μg of thiamine hydrochloride per ml or in M9 minimal medium (47) supplemented with 0.2% glucose. For selection with the antibiotic trimethoprim, Iso-Sensitest broth and Iso-Sensitest agar (Oxoid) were used. When appropriate, antibiotics were added in the following concentration: ampicillin (sodium salt), 100 μg/ml; chloramphenicol, 10 μg/ml; tetracycline hydrochloride, 10 μg/ml; kanamycin sulfate, 30 μg/ml; and trimethoprim, 100 μg/ml. M13mp18/19 and its derivatives were propagated in *E. coli* XL-1 Blue (45, 79). The plasmids used in this study are listed in Table 1.

Conjugations. Entry exclusion experiments were performed as quantitative filter mating assays. Appropriate amounts of donor (0.5 ml, A_{600} = 0.5) and respective recipient cells (5.0 ml, A_{600} = 0.5) were mixed and collected onto a Millipore filter (pore size, 0.45 μm; diameter, 25 mm). The filter was incubated for 1 h at 37°C on a nutrient agar plate without selection. Cells were resuspended and transconjugants were selected on M9 minimal medium plates containing the appropriate antibiotic, i.e., kanamycin sulfate (30 μg/ml) for RP4, R702, RSF1010K, and pVWDG23110Δ0.2; trimethoprim (100 μg/ml) for R751, and tetracycline hydrochloride (10 μg/ml) for pLG273.

DNA techniques. Standard molecular cloning techniques were performed according to Sambrook et al. (58).

Generation of *trbK* mutants. The structural *trbK* gene was subcloned in M13mp19. Single-stranded DNA was isolated by standard techniques (58). Mutagenesis was performed according to the oligonucleotide-directed in vitro method of Sayers et al. (60). The oligonucleotide GTTGTCGCCGCCTGCCA

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TABLE 1. Plasmids used in this study

Plasmid	Description	Relevant genotype	Selective marker(s)[a]	Replicon	Reference or source
pBR329	Cloning vector		Ap, Cm, Tc	pMB1	11
pDB17HE	Cloning vector; pMS119HEΩ[pDB173 *Xba*I-*Bam*HI, 2.4 kb]; P_{tac}/*lacI*^{q}		Ap	pMB1	51
pDB27	pDB17HEΔ[*Nde*I-*Kpn*I]Ω[RP4 27.450-27.681 kb[b]]	*trbK*$^+$	Ap	pMB1	22
pDB270	pGZ119HEΩ[pDB27, *Hin*dIII-*Eco*RI, 0.3 kb]	*trbK*$^+$	Cm	ColD	22
pGZ119EH,HE	Cloning vector; P_{tac}/*lacI*^{q}		Cm	ColD	39
pJF119EH	Cloning vector; P_{tac}/*lacI*^{q}		Ap	pMB1	19
pJH252	pGZ119EHΩ[pLG252 *Eco*RI, 3.5 kb]	*eex*$^+$	Cm	ColD	This work
pJH52	pDB27Ω[pTrxFus, 2.724-3.080 kb]	*trxA-trbK*$^+$	Ap	pMB1	This work
pLG252	pBR325Ω[ColIb *Eco*RI, 37.5-41 kb]	*eex*$^+$	Ap, Tc	pMB1	10
pLG273	ColIb*drd-1* cib::Tn*10*		Tc	$IncI_1$	31
pML100	pJF119EHΩ[RP4 *Eco*47III-*Eco*47III, 45.871-48.933[b]]	*traF*$^+$ *traG*$^+$	Ap	pMB1	41
pML123	pGZ119EHΔ[*Eco*RI-*Bam*HI]Ω[*Eco*RI-*Xmn*I adapter, RP4 *Xmn*I-*Not*I, 18.841-30.042 kb[b]]	*trbB-trbM*$^+$	Cm	ColD	41
pMS119EH,HE	Cloning vector, P_{tac}/*lacI*^{q}		Ap	pMB1	64
pTrxFus	Fusion vector	*trxA*$^+$	Ap	ColEI	37
pVWDG23110Δ0.2	pBR329Δ[*Pst*I-*Bam*HI, 2.755-0.606 kb]Ω[RP4 *Pst*I-*Bfa*I, 37.942-53.462 kb[b]]	*traA-traM*$^+$	Km	pMB1	22, 39
R702			Km, Tc, Sm, Su, Hg	IncPα	29
R751			Tp	IncPβ	34
RP4			Ap, Km, Tc	IncPα	13, 52
RSF1010K	RSF1010Δ[*Hpa*I-*Eco*RV, 1-1.705 kb]Ω[Tn*903*, 0.878-2.217 kb]		Km	IncQ	41

[a] Ap, ampicillin; Cm, chloramphenicol; Tc, tetracycline; Km, kanamycin; Sm, streptomycin; Su, sulfonamide; Tp, trimethoprim; Hg, mercury.
[b] RP4 sequence coordinates of inserted fragment are given according to published sequence data (52).

GGC (corresponding to RP4 coordinates 27,505 to 27,524) (52) was used to change cysteine-23 to glycine (C-23→G) in mutant *trbK*C23G. The oligonucleotide GCTGGGCTAGAAGCCTCCCGG (corresponding to RP4 coordinates 27,621 to 27,641) (52) was used to introduce an amber codon at the lysine codon 62 in mutant *trbK*K62★. The star indicates truncation of the *trbK* gene product. Base exchanges are in boldface type. DNA sequencing was carried out with T7 Sequenase (Pharmacia) and M13 universal primers to verify the nucleotide sequence of the entire mutant *trbK* genes.

Identification and analysis of proteins. IPTG (isopropyl-β-D-thiogalactopyranoside)-dependent overproduction of proteins was performed as described previously (41). The different forms of wild-type and mutant TrbK proteins were analyzed by using tricine-sodium dodecyl sulfate (SDS)-polyacrylamide gels of the composition 17% T and either 2.6 or 5% C. T denotes the total percent concentration of both monomers (acrylamide and bisacrylamide), and C denotes the percent concentration of the cross-linker relative to the total concentration of T (30, 61). Solid-phase immunoassay was carried out as described before (39, 69), except that separated proteins from tricine gels were transferred overnight onto polyvinylidene difluoride membranes (Immobilon-P; Millipore). Cross-reactions were visualized by using the FluorImager575 and ImageQuaNT software (version 4.1b, Molecular Dynamics).

Purification of TrbK for antiserum preparation. The processed form of TrbK, designated TrbK*, was purified by using strain SCS1(pJH52) (Table 1), which overproduces an N-terminal fusion protein of *E. coli* thioredoxin (Trx) and TrbK. Analysis of crude cell extracts from this strain showed that three polypeptides are visible upon induction with IPTG, corresponding to the unprocessed form of TrxTrbK, to Trx extended by the TrbK signal peptide, TrxTrbK^s, and to the processed form of TrbK, TrbK* (data not shown). The latter polypeptide was purified as follows. A 1.2-liter culture of SCS1(pJH52) was grown to an A_{600} of 0.8. IPTG was added to a final concentration of 1 mM, and shaking was continued for 4 h. The cells were then harvested by centrifugation, resuspended in 25 ml of spermidine solution (100 mM spermidine · 3HCl, 200 mM NaCl, 2 mM EDTA), and frozen in liquid nitrogen. Cells were thawed and 15 ml of 10% sucrose–100 mM Tris-HCl (pH 7.6), 0.5 ml of lysozyme (50 mg/ml), 1 ml of 10% Brij 58, 20 ml of 5% sucrose–100 mM NaCl–50 mM Tris-HCl (pH 7.6), and 15 ml of 5 M NaCl were added; the suspension was kept on ice for 60 min and then centrifuged at 100,000 × *g* for 90 min. The protein was solubilized from the resulting pellet fraction by two sequential extractions in 4.5 M urea–50 mM NaCl–1 mM dithiothreitol–1 mM EDTA–20 mM Tris-HCl (pH 7.6). The combined urea supernatants were saturated with $(NH_4)_2SO_4$ to 60%, stirred for 2 h, and then kept overnight at 4°C. Precipitated proteins were collected by centrifugation and resuspended in 6 M urea–50 mM sodium phosphate (pH 8.0)–300 mM NaCl. TrxTrbK and TrxTrbK^s were resolubilized whereas TrbK* remained in the pellet, which was subsequently resuspended in SDS cracking buffer (15% [wt/vol] glycerol, 5% [wt/vol] SDS, 1 M 2-mercaptoethanol, 100 mM Tris-HCl [pH 6.9]). This TrbK solution in SDS cracking buffer was applied onto a preparative 20% polyacrylamide gel. Denatured TrbK* was purified electrophoretically by using a model 491 Prep Cell (Biorad) according to the manufacturer's instruction and used to raise antibodies in a rabbit.

Membrane fractionation. The separation of cytoplasmic and outer membrane fractions was based on the isopycnic sucrose density centrifugation of the total membrane fraction (50) after mechanical disruption of the cells with a French press (62) and lysozyme treatment. A 0.5-liter culture of SCS1 cells containing the appropriate plasmid was grown to an A_{600} of 0.8 to 1.0. Cells were harvested by centrifugation, resuspended in 20 ml of 10 mM Tris-HCl (pH 7.4)–1 mM $MgCl_2$, and kept on ice during further treatment. RNase A and micrococcal nuclease were added, and cells were broken by three passages through a precooled French pressure cell at 8,000 lb/in^2. The disrupted cells were treated with lysozyme (0.1 mg/ml of cell suspension) for 30 min on ice. After the intact cells were removed by centrifugation, KCl was added to the supernatant to a final concentration of 0.2 M (14), and the membranes were separated from the soluble fraction by centrifugation at 100,000 × *g* for 2 h. The supernatant representing the soluble fraction (cytoplasm and periplasm) was kept and stored at −20°C. The membrane containing pellet was resuspended in 2.5 ml of 10 mM Tris-HCl (pH 7.4). A 1-ml aliquot of this suspension was layered on top of a discontinuous sucrose density gradient consisting of 1.5 ml of 55% and 2 ml (each) of 50, 45, 40, 35, and 30% (wt/wt in 10 mM Tris-HCl [pH 7.4]) sucrose. The gradient was centrifuged for 16 h at 35,000 rpm in a Beckman SW41 rotor at 4°C. Fractions of 1.2 ml were collected starting from the bottom of the gradient. The refractive index of the fractions was measured, and the protein content was determined by the Bradford assay (8).

Mass spectrometric peptide mapping analysis. (i) On-membrane digestion and extraction of peptides. The method described here was adapted and slightly modified from the method described by Pappin et al. (53). A digest buffer consisting of 50 mM ammonium hydrogen carbonate (analytical grade; Merck), 1% 1-*O*-octyl-β-D-glucopyranoside (Aldrich, Milwaukee, Wis.), and 48 ng of trypsin (sequencing grade-modified bovine trypsin; Boehringer Mannheim Biochemica) per μl was freshly prepared. Sulforhodamine B-stained polyvinylidene difluoride membranes with electroblotted TrbK* from SDS-polyacrylamide gel electrophoresis (PAGE) gels were washed with double-distilled water and dried with a Speed Vac evaporator. Bands were excised and cut into small pieces of about 1 square millimeter. The membrane pieces were soaked with a slight excess of the digest buffer (15 to 20 μl) in a 1.5-ml tube such that all pieces were seen to be wet. The membrane pieces were collected by centrifugation. An approximately 0.5-cm-long piece of an aerosol protection filter (for a Gilson P5000 Pipetman) was cut off, attached to the inside of the tube's seal, and wetted with 250 μl of double-distilled water. This enabled vapor to saturate the air in the tube, preventing the membrane pieces from drying out. The sealed tube was incubated at 37°C overnight (18 h) with a thermomixer (Eppendorf) covered with aluminum foil to ensure constant temperature in all parts of the tube. After drying the membrane pieces in a Speed Vac evaporator, tryptic peptides were extracted three times with 200 μl of an extracting solvent consisting of ethanol-formic acid (1:1 [vol/vol]). At each extraction step the membrane pieces were

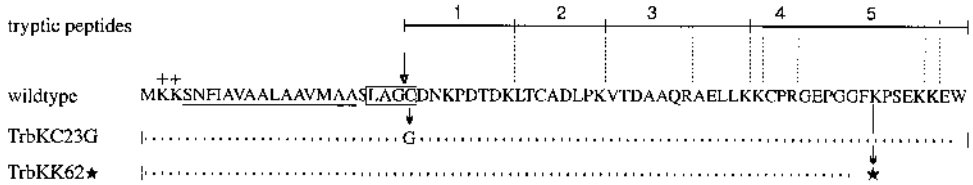

FIG. 1. Amino acid sequence of TrbK deduced from the nucleotide sequence (52). Positively charged residues at the N terminus of the lipoprotein signature are indicated by "+", the hydrophobic core region is underlined, and the lipid attachment motif is boxed. The arrow above the sequence indicates the proposed cleavage site. Mutations in *trbKC*23G and *trbK*K62★, resulting from single base pair exchanges, are shown. ★, truncation of the *trbK* gene product.

allowed to stand in the extracting solvent for 30 min. Extracts were pooled and lyophilized.

(ii) Mass spectrometry. Matrix-assisted laser desorption-ionization–time-of-flight mass spectrometric (36, 66) analyses were performed with a Bruker Reflex-II time-of-flight spectrometer (Bruker-Franzen, Bremen, Germany) equipped with an UV-nitrogen laser (337 nm), a delayed extraction ion source (70), a gridless two-stage potential reflectron, and two dual microchannel plate detectors. The methanol-extracted partially purified TrbK* from the ammonium sulfate precipitate (see Materials and Methods, purification of TrbK*) or the lyophilized extract of the TrbK* tryptic peptide mixture was redissolved in 0.1% trifluoroacetic acid-acetonitrile (2:1 [vol/vol]). Aliquots of 0.4 μl of this solution were combined with equal volumes of saturated 4-hydroxy-α-cyanocinnamic acid (Sigma) (6) in the trifluoroacetic acid-acetonitrile and mixed on the multiple-sample probe tip. Spectra were recorded after evaporation of the solvent and were processed by using the X-MASS data system. Angiotensin-II (Bachem, Heidelberg, Germany) and adrenocorticotropic hormone fragment 18 to 39 (Sigma) were used for internal and external calibration, respectively. When operating in linear mode, the acceleration voltage was set to 30 kV. However, high resolution ($m/\Delta m > 5{,}000$) was obtained in delayed extraction reflectron time-of-flight mode utilizing an extraction potential of 5 to 7 kV during a delay of 360 ns after which an acceleration voltage of 22 kV was applied.

RESULTS

Processing at the N terminus of TrbK. The entry exclusion gene of RP4, *trbK*, has been suggested to encode a lipoprotein (22, 40). Figure 1 shows the amino acid sequence of TrbK deduced from the nucleotide sequence (40). The characteristic features of the signal peptide of lipoproteins are positively charged residues at the N terminus, a hydrophobic core region, and the lipid attachment motif (Fig. 1) (71). It has been shown previously that *trbK* encodes a protein with a molecular mass of approximately 7 kDa (22). A second polypeptide corresponding to the processed form of TrbK was not detected by conventional SDS-PAGE because of the small size of the protein. The first evidence for the actual processing of the N terminus was obtained by analysis of SDS-cell extracts from strain SCS1(pJH52) (Table 1), which encodes a fusion protein of approximately 21 kDa consisting of *E. coli* Trx and TrbK. Three polypeptides are visible upon induction with IPTG, corresponding to the unprocessed form of TrxTrbK (21 kDa), Trx extended by the TrbK signal peptide, TrxTrbK^s (15 kDa), and the processed form of TrbK, TrbK* (5.5 kDa). Thus, N-terminal fusion to Trx did not affect processing of TrbK (data not shown). The 5.5-kDa polypeptide (TrbK*) was purified and used to raise antibodies in rabbits (see Materials and Methods).

The N terminus of the purified TrbK* was analyzed by microsequencing. No amino acid could be identified in the first step during Edman degradation. In the following steps the sequence DNKPDTD was obtained, which coincides with the proposed amino acid sequence for the mature polypeptide of TrbK, starting at position +2 after the predicted cleavage site (Fig. 1) (40). The predicted cysteine residue at position +1 of the mature protein, modified or unmodified, would not be identified by the Edman degradation method. According to the proposed pathway for lipoprotein maturation, the diacylglyceryl portion of phosphatidylglycerol is transferred to the thiol group of the cysteine residue at position +1 of the mature polypeptide (59). Cleavage of the signal peptide by lipoprotein peptidase occurs only after this modification of the prolipoprotein (16, 32, 68). Since we did observe cleavage of the protein precursor of TrbK at the predicted site, it seems likely that TrbK is indeed a lipoprotein.

Lipid modification of TrbK residue C-23. Mass spectrometry (see Materials and Methods) was used to determine the N-terminal modification of the processed form of TrbK (TrbK*). The molecular weight of singly charged protonated TrbK* (TrbK*H$^+$) was determined to be 6,004.5 ± 2.0 atomic mass units (amu). The weight difference is 789.6 ± 2.0 amu greater than the theoretical molecular weight of TrbK*H$^+$ (m/z 5,214.9; one disulfidic bond assumed). This difference indicates a modification in the magnitude of a mass increment of a thiol-bounded bis(palmitoyloxy)-propyl group in combination with an N-terminal palmitoyl group. The mass spectrometric peptide mapping analysis indicates the existence of a fatty acid-bound cysteine residue at the N terminus of TrbK*. No unmodified tryptic peptide 1-9 was detected, whereas a well-resolved signal at m/z 1,822.21 ± 0.2 (peptide 1, Table 2) correlates to the peptide 1-9 containing an *S*-[2,3-bis(palmitoyloxy)-propyl]-*N*-palmitoyl-cysteine residue (Pam_3Cys) with a calculated monoisotopic weight of MH$^+$ = 1,824.17 amu, containing at least one additional olefinic bond, which is assumed to be located in one of the three aliphatic chains. The other cysteine-containing tryptic peptides, positions 10-17 (peptide 2) (Table 2, Fig. 1) and 31-33 (peptide 4) (Table 2, Fig. 1), were found to be cross-linked to each other and to themselves as in the case of the peptide 10-17 (Table 2, Fig. 1). It remains to be shown whether the disulfide bridges detected are also present

TABLE 2. Identification of the N-terminal lipid modification of TrbK* by mass spectrometry of tryptic peptides

Peptide[a]	MH$^+$ calculated m/z (monoisotopic)	MH$^+$ from DE-Ref-TOF–MALDI-MS experiment m/z (monoisotopic) ±0.2[b]
$Pam_3$1-9	1824.17	1822.21
10-17 (Cys-12 reduced)	860.46	860.61
18-24	760.40	760.53
25-29		n.d.[c]
30-30		n.d.[c]
10-17-(S-S)-31-33	1232.61	1232.59
10-17-(S-S)-10-17	1717.88	1717.53
34-44	1132.56	1132.56
45-45		n.d.[c]
46-47		n.d.[c]

[a] Amino acid residues of TrbK* 1 to 47, corresponding to positions 23 to 69 of the propolypeptide (see Fig. 1), are given.

[b] DE-Ref-TOF–MALDI-MS, delayed extraction reflectron time-of-flight–matrix-assisted laser desorption-ionization–mass spectrometric; see Materials and Methods.

[c] n.d., not detected. Peptides are not detectable with the applied method (see Materials and Methods).

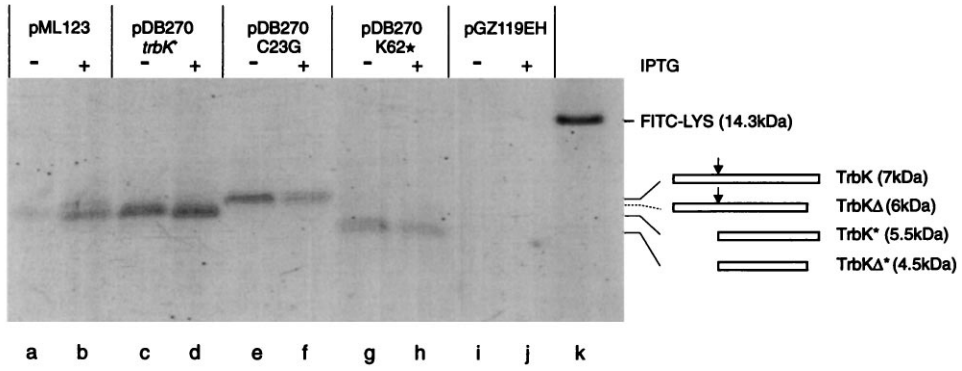

FIG. 2. Immunological identification of TrbK wild-type and mutant proteins. SDS extracts of SCS1 cells containing the indicated plasmids were separated on a polyacrylamide gel (tricine, 17%T and 5%C), electrophoretically transferred to an Immobilon-P membrane, and subsequently incubated with TrbK antiserum (dilution, 1:10,000). Transcription was either induced or noninduced by addition of IPTG, indicated by "+" or "−", respectively. For noninduced cells (lanes a, c, e, g, and i) 2-μl aliquots of each extract were applied, and for induced cells 2 μl of SCS1(pML123) and SCS1(pGZ119EH) (lanes b and j) or 0.1 μl of all other extracts (lanes d, f, and h) were applied. Lane k contains the molecular mass standard fluorescein isothiocyanate-labelled lysozyme (FITC-LYS). Cross-reactions were visualized as described in Materials and Methods. The identities of the different forms of TrbK are indicated on the right: TrbK, unprocessed form of the wild-type polypeptide; TrbKΔ, unprocessed form of the C-terminally truncated polypeptide of TrbK from mutant *trbK*K62★; TrbK*, processed form of the wild-type polypeptide; TrbKΔ*, processed form of the C-terminally truncated polypeptide of TrbK from mutant *trbK*K62★.

in functional TrbK molecules in vivo. Thus, the mass spectrometric analyses clearly showed the lipid modification of cysteine-23, and in addition, verified the predicted primary sequence of TrbK* (40).

Generation of *trbK* mutants. For functional and structural studies of TrbK we constructed two mutants by oligonucleotide-directed in vitro mutagenesis (60) (see Materials and Methods) (Fig. 1). The cysteine in the lipid attachment motif of TrbK was changed to a glycine in mutant *trbK*C23G. Accordingly, modification with diacylglycerol cannot occur, and hence the signal peptide should not be cleaved by the lipoprotein peptidase. To obtain a higher resolution between the unprocessed and the processed form of TrbK, SDS cell extracts were analyzed on a tricine-buffered SDS-polyacrylamide gel (61). Immunological analysis of cell extracts of SCS1(pDB270*trbK*$^+$) (Table 1) and SCS1(pDB270*trbK*C23G) showed that in the latter strain the smaller, processed polypeptide is lacking (Fig. 2, lanes c to f, and see Fig. 4, lanes d and g), indicating that processing is impaired in this mutant.

A second mutant, *trbK*K62★, was constructed by introducing an amber codon at position 62 by using an analogous strategy as that for *trbK*C23G (see Materials and Methods), which resulted in the deletion of eight amino acids at the C terminus (Fig. 1). As shown by immunological analysis of cell extracts of strain SCS1(pDB270*trbK*K62★), the apparent sizes of obtained polypeptides coincided well with the predicted ones (Fig. 2, lanes g and h, and see Fig. 4, lane j).

TrbK localizes mainly to the cytoplasmic membrane. To better understand the mechanism by which TrbK exerts its entry exclusion function, we attempted to determine its localization within the cell envelope. It was known from previous studies that TrbK functions independently of other gene products of the Tra2 operon (22), thus it should be possible to determine its final localization in cells which contain *trbK* in the absence of other Tra2 gene products. Localization of TrbK was analyzed in cells containing *trbK* alone (Table 1, pDB270) and compared with cells carrying plasmid pML123 (Table 1) encoding the whole of the Tra2 core region.

SCS1 cells containing the appropriate plasmid were separated into soluble compartments and cytoplasmic and outer membrane fractions as described in Materials and Methods. To avoid artificial effects by extensive overexpression of TrbK, noninduced cells were used for each of the fractionation experiments. Sucrose density gradient centrifugation of total membrane preparations resulted in four bands after separation (Fig. 3A). Above the middle section of the gradient two slightly yellow stained bands of low density were observed, corresponding to the cytoplasmic membrane. One rather sharp band of high density close to the bottom of the gradient together with a fainter, rather diffuse band just above the latter one represented the outer membrane. Fractions were collected and the density and the protein content were estimated (Fig. 3A). Cytoplasmic and outer membrane fractions were analyzed by SDS-tricine-PAGEs and showed clearly distinguishable protein patterns (Fig. 3B). At this stage of the study we cannot exclude a possible cross-contamination of the membrane fractions. However, the protein pattern and the buoyant densities of the membrane fractions indicate fairly good separation.

To determine the membrane distribution of wild-type and mutant TrbK proteins, solid-phase immunoassay analysis of sucrose gradient fractions of SCS1 cells containing plasmid pML123, pDB27*trbK*$^+$, pDB27*trbK*C23G, or pDB27*trbK*K62★ was carried out as described in Materials and Methods. In each of the tested strains, TrbK or its mutant derivatives were detectable in both membrane fractions (Fig. 3C). However, the portion found in the cytoplasmic fractions 6 to 8 was significantly higher than the portion detected in the outer membrane fractions 1 to 3, i.e., 56 to 66% compared with 11 to 16%, respectively (Fig. 3D).

There was no significant difference in the distribution of the proteins between the overproduced TrbK in pDB27*trbK*$^+$, which contained the T7 *gene10* ribosome binding site, and the TrbK, expressed from its original gene arrangement (pML123), or between wild-type TrbK and the mutant proteins. However, only TrbKC23G was detected in the culture supernatant, and it was also detected in a higher amount in the soluble fraction, representing the cytoplasm and the periplasm, than were the wild-type protein and TrbKK62★ (Fig. 4, lanes b and c, e and f, and h and i, respectively).

The results suggest that (i) TrbK is transferred across the cytoplasmic membrane, in which it remains anchored facing the periplasmic space; (ii) TrbK might also interact directly or via protein-protein interactions with the outer membrane; (iii) although lipoprotein modification is not absolutely required

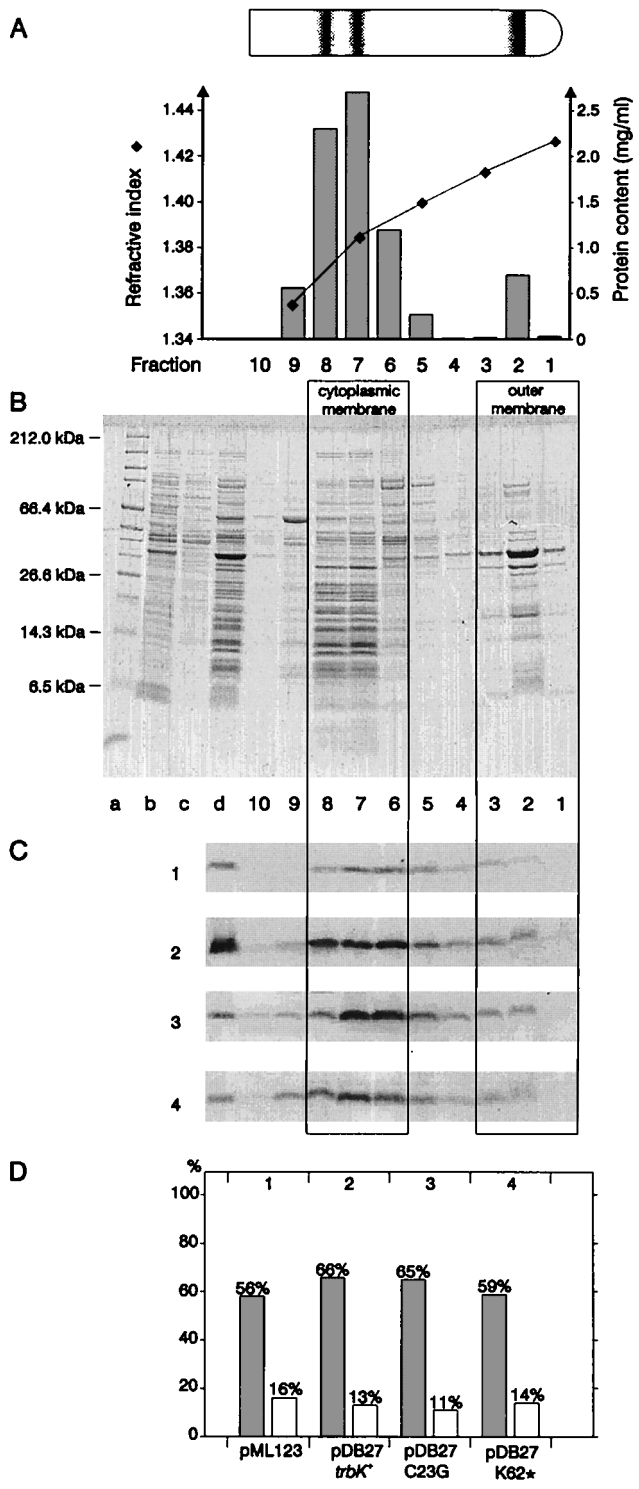

FIG. 3. Detection of wild-type and mutant TrbK proteins in membrane fractions of *E. coli*. (A) Density and protein content of fractions after sucrose density gradient centrifugation (see Materials and Methods) of the total membrane preparation derived from strain SCS1(pDB27*trbK*$^+$). (B) Protein pattern of membrane fractions of strain SCS1(pDB27*trbK*$^+$). Samples were separated on a SDS-tricine-polyacrylamide gel (17%T, 2.6%C) and stained with Coomassie brilliant blue. Fractions representing the cytoplasmic and the outer membranes are boxed. Lane a, broad-range protein marker with the sizes of several reference proteins indicated; lane b, SDS extract of IPTG-induced cells; lane c, soluble fraction; lane d, total membrane fraction. Numbers 10 to 1 designate the fractions from the sucrose density gradient. (C) Immunological identification of wild-type and mutant TrbK proteins in total membrane and sucrose density gradient fractions of SCS1 cells carrying plasmid pML123 (1), pDB27*trbK*$^+$ (2), pDB27C23G (3), or pDB27K62★ (4). The samples applied correspond to the samples from panel B, and cytoplasmic and outer membrane fractions are also indicated. (D) Distribution of wild-type and mutant TrbK proteins within cytoplasmic and outer membrane fractions detected in solid-phase immunoassays. Solid and open bars indicate the percentage of TrbK detected in the cytoplasmic membrane fractions 6 to 8 and the outer membrane fractions 1 to 3, respectively.

for correct localization of TrbK, interaction of the mutant protein TrbKC23G with the membrane seems to be somewhat weaker compared with the wild-type protein under the conditions applied in the experiment; and (iv) apparently, the C-terminal eight amino acid residues are not required for correct localization of TrbK within the cell envelope, but they are essential for function (see below).

The C-terminal region of TrbK but not lipid modification is essential for the RP4 entry exclusion function. A single gene, *trbK*, has been identified to encode the RP4 entry exclusion function (22). The mechanism by which TrbK lowers the transfer frequency of IncP plasmids when present in the recipient cell has not been studied yet. To begin with the analysis of essential amino acid residues and structural requirements for the entry exclusion function of TrbK, we tested the mutants of *trbK*, described above, for their ability to exclude R702, an IncPα plasmid similar to RP4. Filter mating experiments were carried out as described in Materials and Methods. The transfer frequency of R702 is lowered by approximately five orders of magnitude when wild-type *trbK* is present in the recipient cell compared with the transfer frequency when plasmid-free recipients are used (Table 3).

Interestingly, mutant TrbKC23G is also capable of excluding the IncPα plasmid, suggesting that lipoprotein modification is not essential for the entry exclusion function of TrbK. However, the entry exclusion index obtained with this mutant is 10 times lower than that of the wild-type TrbK (Table 3). This might be due to the observed tendency of TrbKC23G to detach from the membrane (Fig. 4). In contrast, mutant TrbKK62★, which lacks eight amino acid residues at the C terminus, was completely impaired for entry exclusion. Exclusion indices obtained in experiments with this mutant were the same as in experiments with recipient cells carrying the vector plasmid pGZ119EH (Table 3). The mutant protein TrbKK62★ seems to be less stable when expressed under the conditions applied, because the amount of protein detected by solid-phase immunoassay in extracts of noninduced SCS1 cells containing plasmid pDB270*trbK*K62★ was lower than in extracts of cells carrying the wild-type plasmid pDB270*trbK*$^+$ (Fig. 2, lanes c and g). However, this observation would not explain the complete inability of the amber mutant to function in entry exclusion. Therefore, we conclude that the eight C-terminal amino acid residues of TrbK play an essential role for entry exclusion.

Specificity of the entry exclusion process mediated by TrbK of RP4. Entry exclusion was defined as the inhibition of plasmid transfer into a recipient cell which already contains the same or a closely related plasmid. We compared the entry exclusion functions of the IncPα plasmid RP4 and the $IncI_1$ plasmid ColIb-P9. The transfer frequency of RP4, but not of pLG273 (Table 1), a ColIb-P9 derivative, was reduced when the recipient cell contained plasmid pDB270 (RP4 *trbK*$^+$) (Table 4). In contrast, recipient cells containing *eex* of ColIb-P9 (pJH252 [Table 1]) excluded pLG273 but not RP4 (Table 4). Hence, as expected, entry exclusion by TrbK is specific for IncP plasmids.

We then compared the entry exclusion ability of TrbK against different IncP plasmids, i.e., RP4 (IncPα), R702

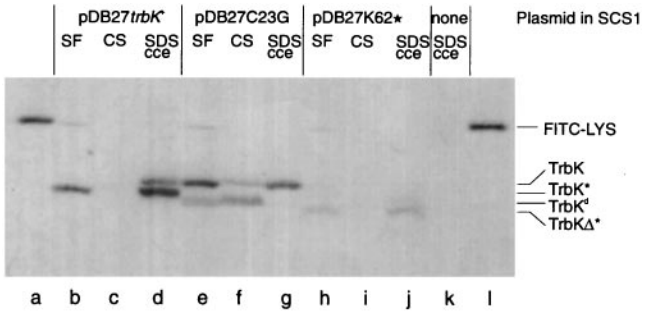

FIG. 4. Detection of wild-type and mutant TrbK proteins in soluble fractions and culture supernatants. Lanes a and 1 contain the molecular mass standard fluorescein isothiocyanate-labelled lysozyme (FITC-LYS). Aliquots from the soluble fraction (SF) (0.3 A_{600} units), from concentrated culture supernatant (CS) 1.5 A_{600} units), and SDS cell extracts (SDS-cce) 0.002 A_{600} units) of SCS1 cells carrying the indicated plasmids were separated on an SDS-tricine-polyacrylamide gel (17%T, 5%C), transferred to an Immobilon-P membrane, incubated with TrbK antiserum (dilution 1:10,000), and visualized as described in Materials and Methods. Culture supernatants were concentrated by using Centricon-33 concentrators (AMICON) with a molecular mass cutoff of 3 kDa according to the manufacturer's instructions. The different forms of wild-type and mutant TrbK proteins are indicated on the right: TrbK and TrbK*, unprocessed and processed forms of the wild-type polypeptide, respectively; TrbK^d, degradation products of the mutant polypeptide derived from strain SCS1(pDB27C23G); TrbKΔ*, processed form of the C-terminally truncated polypeptide derived from strain SCS1(pDB27K62★).

(IncPα), and R751 (IncPβ) (Table 5). Transfer frequencies of the two IncPα plasmids into recipient cells containing plasmid pDB270*trbK*$^+$ are lowered to the same extent (Table 5). Entry of the IncPβ plasmid R751 into the recipient cells is also affected by TrbK of RP4. However, the entry exclusion index for this plasmid is about three times lower compared with those of RP4 and R702 (Table 5). Hence, TrbK cannot prevent entry of R751 with the same efficiency as it prevents entry of RP4. This suggests a greater specificity of entry exclusion by RP4 TrbK against IncPα plasmids.

Entry exclusion by TrbK is independent of the presence of *trbK* in the donor cell. How does TrbK prevent plasmid entry into the recipient cell? One possible mechanism would be that TrbK within the recipient cell interacts directly or indirectly with protein(s) encoded by the donor cell, resulting in inhibition of the DNA transfer. TrbK itself could be a candidate for the interacting protein in the donor cell. Thus, the question arises whether entry exclusion by TrbK functions only within the recipient cell or whether *trbK* has to be present in the donor cell as well. To verify this, filter mating experiments were performed with the two-plasmid system established in previous studies (22, 39). Plasmid pVWDG23110Δ0.2 (Table 1), containing the Tra1 region of RP4, including the transfer origin, *oriT*, was mobilized into the recipient cell by the Tra2 plasmid pML123 (Table 1) or its derivative pML123m*trbK*9, in which a MURFI linker was inserted just after the start codon of *trbK* (22). TrbK is not expressed in this mutant as confirmed by solid-phase immunoassay (data not shown). Since *trbK* is not essential for RP4 transfer, plasmid pML123m*trbK*9 efficiently mobilizes pVWDG23110Δ0.2 (22) (Table 6). The recipient strain W2438 contained either no plasmid or pDB270*trbK*$^+$. Entry exclusion indices obtained with pML123 and pML123m*trbK*9 in the donor cell were essentially the same (Table 6), suggesting that for the entry exclusion function *trbK* acts only in the recipient cell.

Does TrbK interact with a gene product encoded by the RP4 Mpf system? Depending on possible mechanisms by which TrbK exerts its entry exclusion function, any component, with the exception of *trbK* (see previous paragraph and Table 6), of the transfer apparatus encoded by plasmids pVWDG23110Δ0.2 and pML123 (Table 1) could be the target of TrbK in the donor cell. We attempted to further specify the donor-encoded component(s) in the entry exclusion process. To rule out the possibility of specific interaction of TrbK with the transferred DNA strand or with a component(s) of the relaxosome (TraI,

TABLE 3. Entry exclusion of the IncPα plasmid R702 by *trbK* (RP4) mutants

Plasmid in recipient strain[a]	Transfer frequency[b]	Eex index[c]	Eex phenotype[d]
No plasmid	1	1	−
pGZ119EH	6.0×10^{-1}	1.7	−
pDB270	7.2×10^{-6}	140,000	++
pDB270*trbK*C23G	6.1×10^{-5}	16,000	+
pDB270*trbK*K62★	6.0×10^{-1}	1.7	−

[a] *E. coli* W2438 served as recipient strain in all experiments.
[b] Transconjugants per donor cell after a 1-h mating of the donor, W3110 T$^-$ (R702), with the indicated recipients at 37°C as described in Materials and Methods. The given frequencies represent the average values of three independent experiments.
[c] The Eex index is calculated by dividing the transfer frequency for recipient strain W2438 containing no plasmid with the frequency of W2438 containing the plasmid under study.
[d] ++, +, and − indicate Eex indices of $>10^5$, 10^4 to 10^5, and <10, respectively.

TABLE 4. Specificity of entry exclusion by RP4 *trbK* for IncP plasmids

Plasmid in donor strain HB101	Plasmid in recipient strain W2438	Transfer frequency[a]	Eex index[b]	Eex phenotype
RP4	None	1.1	1.0	−
RP4	pGZ119EH	1.9	0.6	−
RP4	pDB270*trbK*$^+$	1×10^{-5}	110,000	+
RP4	pJH252*eex*$^+$ (IncI$_1$)	1.5	0.7	−
pLG273	None	1.2	1.0	−
pLG273	pGZ119EH	1.4	0.9	−
pLG273	pDB270*trbK*$^+$	1.2	1.0	−
pLG273	pJH252*eex*$^+$ (IncI$_1$)	9×10^{-4}	1,330	+

[a] Transconjugants per donor cell after a 1-h mating of donors with the indicated recipients at 37°C as described in Materials and Methods. The given frequencies represent the average values of three independent experiments.
[b] The Eex index is calculated by dividing the transfer frequency for recipient strain W2438 containing no plasmid with the frequency of W2438 containing plasmid pDB270.

TABLE 5. Comparison of entry exclusion by RP4-TrbK against IncPα and IncPβ plasmids

Plasmid in donor strain HB101	pDB270*trbK*$^+$ in recipient W2438	Transfer frequency[a]	Eex index[b]
RP4 (IncPα)	−	6.0×10^{-1}	1
RP4 (IncPα)	+	4.5×10^{-6}	130,000
R702 (IncPα)	−	9.0×10^{-1}	1
R702 (IncPα)	+	6.0×10^{-6}	150,000
R751 (IncPβ)	−	2.0×10^{-1}	1
R751 (IncPβ)	+	4.5×10^{-6}	44,000

[a] Transconjugants per donor cell after a 1-h mating of donors with the indicated recipients at 37°C as described in Materials and Methods. The given frequencies represent the average values of three independent experiments.
[b] The Eex index is calculated by dividing the transfer frequency for recipient strain W2438 containing no plasmid with the frequency of W2438 containing plasmid pDB270.

TABLE 6. Entry exclusion by TrbK is independent of the presence of *trbK* in the donor cell

Plasmids in donor strain HB101	pDB270*trbK*$^+$ in recipient strain W2438	Transfer frequency[a]	Eex index[b]
pVWDG23110Δ0.2, pML123	−	1	1
pVWDG23110Δ0.2, pML123	+	3.3×10^{-6}	300,000
pVWDG23110Δ0.2, pML123m*trbK9*	−	1	1
pVWDG23110Δ02, pML123m*trbK9*	+	3×10^{-6}	330,000

[a] Transconjugants per donor cell after a 1-h mating of donors with the indicated recipient cells at 37°C as described in Materials and Methods. The given frequencies represent the average values of three independent experiments.

[b] The Eex index is calculated by dividing the transfer frequency for recipient strain W2438 containing no plasmid with the frequency of W2438 containing plasmid pDB270.

TraJ, TraK, and TraH encoded by the RP4 Tra1 region), we tested the ability of TrbK to exclude the non-self-transmissible IncQ plasmid RSF1010, which contains a transfer origin (*oriT*) and encodes its own relaxosomal components (15). The RP4 Tra2 core region together with *traF* and *traG* of the RP4 Tra1 region was shown to be sufficient for mobilization of RSF1010 (41). We measured the mobilization frequencies of a kanamycin-resistant derivative of RSF1010 by plasmids pML123, containing Tra2, and pML100 (Table 1), carrying *traF* and *traG*. The recipient strain W2438 contained either no plasmid, plasmid pDB270*trbK*$^+$, or the vector plasmid pGZ119EH. It was found that RSF1010 is excluded from recipient cells containing *trbK* to about the same extent as the RP4 Tra1 plasmid pVWDG23110Δ0.2 (compare Tables 6 and 7). This demonstrates that TrbK does not specifically recognize the single-stranded DNA molecule.

Further, RSF1010 is excluded by *trbK* but not by *eex* of the IncI plasmid ColIb-P9 when mobilized by the Mpf complex of RP4 (Table 7). In contrast, transfer frequencies of RSF1010 are reduced by *eex*, but not by *trbK*, when it is mobilized by the IncI plasmid pLG273 (Table 7). This indicates that for entry exclusion of RSF1010 a homologous Mpf system is required in the donor cell. Therefore, we suggest that TrbK interacts directly or indirectly with a component(s) of the RP4 Mpf system (Tra2 plus TraF) or TraG, inhibiting a critical step of the DNA transfer process.

DISCUSSION

Transfer systems of conjugative plasmids, especially of broad-host-range plasmids, are often very efficient. However, continuous mating of cells containing an identical or closely related plasmid is energy consuming and could cause damage to cells within mating aggregates. Thus, many conjugative plasmids have evolved entry exclusion systems to reduce DNA transfer between two donor cells. The entry exclusion functions of the characterized plasmids are encoded by their transfer operons, but they are not essential for transfer (2, 20, 22, 76).

At least two different entry exclusion strategies exist. One is the surface-oriented inhibition of stable mating pair formation. TraT of the F plasmid is the best studied member of this group. TraT is a surface-exposed outer membrane lipoprotein (1, 54). It was thought to disturb the interaction of the sex pilus tip with the proposed cell surface receptor, OmpA, in *E. coli* (57, 63). However, the main and original function of TraT might be to mediate greater serum resistance to F-containing cells (65). The entry exclusion systems of the pheromone-inducible plasmids pAD1 and pCF10 of gram-positive bacteria (18, 49, 73) probably belong to the same group of surface-mediated exclusion systems.

A different, probably larger group of entry exclusion functions is mediated by proteins which localize to the cytoplasmic membranes of gram-negative bacteria. It has been shown for TraS of the F plasmid and for ExcA of the IncI plasmid R144 that they function after stable mating pairs have been established. These proteins are thought to inhibit DNA entry into the cell (1, 28). According to our results presented here, we include the entry exclusion function of the IncP plasmids, encoded by *trbK*, into this group of functionally similar proteins.

As we demonstrated in this study, TrbK is anchored to the cytoplasmic membrane by its lipid moiety, facing the periplasmic space. From analysis of the proposed amino acid sequence it was already assumed that TrbK localizes to the cytoplasmic membrane because it contains an aspartate at position +2 of the mature polypeptide (40). The amino acid residue at this position was thought to function as a sorting signal for lipoprotein localization (21, 78). Lipoprotein modification and cleavage of the signal peptide of this hydrophilic protein was shown to be nonessential for the entry exclusion function. However, interaction between the cytoplasmic membrane and the lipid moiety of wild-type TrbK is stronger than the interaction between the membrane and the moderate hydrophobic signal peptide in mutant TrbKC23G. Hence, the lipoprotein modification might stabilize the integration of TrbK into the membrane and optimize possible functional interactions with other proteins. Since there is evidence that TrbK probably interacts directly or indirectly with components of the RP4 Mpf system of the donor cell, we assume that TrbK functions after the cell-to-cell contact has been established.

How does TrbK prevent entry of the single-stranded DNA molecule into the cell? The following model should help to explain possible mechanisms of entry exclusion mediated by TrbK of RP4. A donor cell recognizes a potential recipient cell

TABLE 7. Entry exclusion of RSF1010

Plasmids in donor strain HB101	Plasmid in recipient strain W2438	Mobilization frequency[a]	Eex index[b]	Eex phenotype
RSF1010K, pML123, pML100	None	1.5	1	−
RSF1010K, pML123, pML100	pDB270*trbK*$^+$	1.4×10^{-5}	110,000	+
RSF1010K, pML123, pML100	pJH252*eex*$^+$ ($IncI_1$)	0.9	1.7	−
RSF1010K, pLG273	None	4×10^{-2}	1	−
RSF1010K, pLG273	pDB270*trbK*$^+$	2.6×10^{-2}	1.5	−
RSF1010K, pLG273	pJH252*eex*$^+$ ($IncI_1$)	2.3×10^{-5}	1,130	+

[a] Transconjugants per donor cell after a 1-h mating of donors with the indicated recipients at 37°C as described in Materials and Methods. The given frequencies represent the average values of three independent experiments.

[b] The Eex index is calculated by dividing the mobilization frequency of RSF1010K into recipient strain W2438 containing no plasmid with the frequency for W2438 containing the plasmid under study.

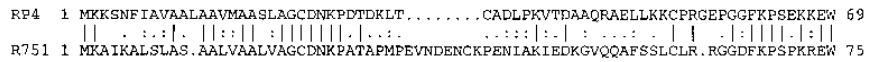

FIG. 5. Comparison of *trbK* gene products of RP4 and R751. Amino acid sequences deduced from nucleotide sequences were compared by using the BESTFIT algorithm (17).

probably by an initial contact of the conjugative pilus. After the cell-to-cell contact has been established, there are certainly a number of steps necessary to prepare the recipient cell for the uptake of the single-stranded DNA. Some sort of hole or pore or channel has to be formed through the cell envelope of the recipient spanning the outer membrane, the periplasmic space including the peptidoglycan layer, and the cytoplasmic membrane. If certain sites were preferred for entry, some sort of receptor recognition would be involved. The whole process is thought to require a number of enzymatic and binding activities. These functions are probably encoded by the Mpf system of the donor cell, but they have not yet been studied in detail.

The expression of TrbK is tightly associated with the expression of the components of the Mpf system encoded by the Tra2 region of RP4 (40, 41). However, TrbK is not essential for transfer (22). In addition, we have shown that for entry exclusion TrbK functions only at the recipient's side of the mating pair. We have also shown that inhibition of DNA transfer by TrbK depends on the presence of the RP4 Mpf system, Tra2 plus TraF, and TraG in the donor cell. Entry exclusion by TrbK is independent of the type of the transferred single-stranded DNA molecule and of the components involved in formation of the relaxosome. From our data we assume that TrbK inhibits one of the proposed steps in formation of the mating pore within the recipient cell. One possibility would be that TrbK inhibits the enzymatic action of an Mpf component at the cell envelope of the recipient. Since it has been shown previously that efficiency of transfer inhibition by TrbK is dosage dependent (22), it is also possible that TrbK masks or alters a receptor site within the cytoplasmic membrane of the recipient cell. Both direct and indirect interactions can be assumed.

The entry exclusion mechanism of RP4 and perhaps also of other conjugative plasmids might have some analogy to the superinfection exclusion process of T-even-type coliphages (for a review, see reference 43). Bacteriophage T4 encodes two proteins, Imm and Sp, localizing to the cytoplasmic membrane, which contribute independently to the inhibition of superinfection of *E. coli* cells by T-even phages. After normal binding of T4 to the cell, Imm blocks the DNA transfer across the cytoplasmic membrane, probably by interacting with a receptor protein. Sp was found to inhibit local degradation of bacterial murein by a phage-associated lysozyme (43).

The question arises which component of the Mpf system of RP4 is the target for TrbK action. The entry exclusion process is very specific. TrbK does not inhibit DNA transfer mediated by an unrelated conjugative system, as demonstrated in this study for the $IncI_1$ plasmid ColIb-P9. We have also shown that RP4 TrbK expresses a higher specificity towards IncPα plasmids than towards the IncPβ plasmid R751. The organizations of the transfer regions of RP4 and R751 are homologous. Extensive sequence similarities of corresponding gene products have been identified (46, 72, 80). It was shown previously that the Tra2 regions of RP4 and R751 are interchangeable (39). The similarities between the amino acid sequences of individual Tra2 gene products of RP4 and R751 range from about 75 to 92% (67). In contrast, the products of the RP4 and R751 *trbK* genes show only 41% identity and 60% similarity (67). The highest degree of similarity between these two homologous gene products was found at the N terminus, which contains the lipoprotein signature, and at the C terminus. The amino acid sequence of the central region of the predicted polypeptides was found to be quite divergent (Fig. 5). However, both genes encode small hydrophilic lipoproteins. The differences in the primary sequence of the two gene products might reflect their greater specificity toward the homologous plasmid. Therefore, the lower exclusion efficiency of R751 by RP4 TrbK might be due to its inefficient interaction with the corresponding Mpf gene product of R751 within the donor cell. In summary, the target for TrbK might consist of an Mpf component, which is much less conserved than the majority of the Mpf proteins. The identification of this component will be a subject of future studies.

The C-terminal region of TrbK is probably directly involved in the interaction of TrbK with the corresponding Mpf component of the donor cell, since deletion of this region resulted in a complete loss of functional activity. This rather drastic change of the very small protein might have also resulted in a structure alteration of the protein. Single amino acid residue substitutions within this region will help to clarify the observed effect.

Recently, the sequence of the operons responsible for the conjugative DNA transfer of the *Agrobacterium tumefaciens* Ti plasmid was published (4). Among IncP-related conjugation systems the gene products of the Ti Tra3 region and the IncP Tra2 region share the highest similarity. There is one exception, TrbK, for which the lowest degree of conservation was observed. Interestingly, the Ti Tra3 TrbK sequence does not possess the typical lipoprotein signature (4). However, in this work we could show that the lipid modification of RP4 TrbK is not essential for its entry exclusion function. It will be interesting to learn whether the Ti *trbK* encodes the entry exclusion or another function of the Ti conjugative transfer system.

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