JOURNAL OF BACTERIOLOGY, June 1991, p. 3872–3878
0021-9193/91/123872-07$02.00/0

Vol. 173, No. 12

Characterization of *trbC*, a New F Plasmid *tra* Operon Gene That Is Essential to Conjugative Transfer

SUMIT MANEEWANNAKUL, KESMANEE MANEEWANNAKUL, AND KARIN IPPEN-IHLER*

Department of Medical Microbiology and Immunology, Texas A&M University, College Station, Texas 77843

Received 21 December 1990/Accepted 8 April 1991

We have characterized a previously unidentified gene, *trbC*, which is contained in the transfer region of the *Escherichia coli* K-12 fertility factor, F. Our data show that the *trbC* gene is located between the F plasmid genes *traU* and *traN*. The product of *trbC* was identified as a polypeptide with an apparent molecular weight (M_a) of 23,500 that is processed to an M_a-21,500 mature protein. When ethanol was present, the M_a-23,500 polypeptide accumulated; the removal of ethanol resulted in the appearance of the processed mature protein. Subcellular fractionation experiments demonstrated that the processed, M_a-21,500 mature protein was located in the periplasm. DNA sequence analysis showed that *trbC* encodes a 212-amino-acid M_r-23,432 polypeptide that could be processed to a 191-amino-acid M_r-21,225 mature protein through the removal of a typical amino-terminal signal sequence. We also constructed two different Km^r gene insertion mutations in *trbC* and crossed these onto the transmissible F plasmid derivative pOX38. We found that cells carrying pOX38 *trbC* mutant plasmids were transfer deficient and resistant to infection by F-pilus-specific phages. Transfer proficiency and bacteriophage sensitivity were restored by complementation when a *trbC*$^+$ plasmid clone was introduced into these cells. These results showed that *trbC* function is essential to the F plasmid conjugative transfer system and suggested that the TrbC protein participates in F-pilus assembly.

Previous analyses have led to the identification of a large number of F plasmid genes that are required for conjugative transfer of the *Escherichia coli* fertility factor, F (for a review see references 16 and 38). All of these genes are clustered within a 33-kb segment of F known as the transfer (*tra*) region, and the majority are included in a lengthy operon regulated by the positive control product of *traJ*. Characterization of F*lac tra* mutant plasmids has classified the *tra* operon genes into four functional groups that include genes required for (i) the synthesis and assembly of F pili, filaments thought to be important in establishing contact between donor and recipient cells; (ii) the stabilization of these contacts; (iii) the conjugative DNA metabolism necessary for nicking, unwinding, and transporting a single strand of F DNA into the recipient; and (iv) the synthesis of surface exclusion proteins that block the entry of additional F plasmids into F donor cells.

To identify the protein activities involved in conjugative transfer functions, our laboratory has been characterizing the genes and gene products encoded by the central segment of the F *tra* operon. For this purpose, we have been analyzing the protein products and *tra* mutant-complementing activities expressed by plasmids carrying cloned F DNA fragments. Such studies have revealed that the F *tra* region contains a number of genes that had not been identified by earlier mutational analyses (9, 31, 39, 41). Since mutants containing defects in these genes had not been isolated, the requirement for these genes in conjugative processes has remained to be demonstrated. In this report, we present data characterizing the location, nucleotide sequence, and product of the new *tra* operon locus, *trbC*. Through the construction and characterization of *trbC* mutants, we also show that the TrbC product is an essential component in the F plasmid conjugative transfer system.

* Corresponding author.

MATERIALS AND METHODS

Bacterial strains and plasmids. Table 1 summarizes the construction of the plasmids carrying the F *tra* region DNA segments that were used in this study. The segments of the *tra* region that these plasmids carry are diagrammed in Fig. 1. The Tra$^+$ F plasmid derivatives pOX38 and pOX38-Km were obtained from R. Deonier using strain RD17 (37). Plasmids pUC4K and pUC4-KISS were purchased from Molecular Biology Division, Pharmacia Inc., Piscataway, N.J., and used as a source of restriction fragments containing the Tn*903* kanamycin resistance (Km^r) gene. Plasmid vectors used in cloning included pACYC177 (4), pBluescript/KS+ (pBS/KS+; Stratagene, La Jolla, Calif.), and pTZ18U (26; Chemical Division, Bio-Rad, Richmond, Calif.). In addition, vector pKI497 was derived by insertion of an end-filled *Eco*RI-*Ava*I fragment from pBR322 (36) into *Pvu*II-digested pTZ18U DNA. This process replaced pTZ18U *lacZ*α sequences with *tcy*. Thus, pKI497 is a 3.9-kb vector that carries ampicillin resistance (Amp^r) and tetracycline resistance (Tc^r) as well as the bacteriophage f1 origin of replication carried by pTZ18U. Plasmid pS2 was constructed by cloning the 2.5-kb *Pst*I-*Eco*RV (*phoA*) fragment from pCH39 (14) into the *Pst*I and *Eco*RV multicloning *lacZ*α sites of pBS/KS+; pS2 DNA was then used as the source of *phoA* cassette DNA in protein fusion constructs.

The origins of strains XK1200 [F$^-$ *lac*Δ*U124* Δ(*nadA gal attλ bio*) *gyrA*], XK5456 [F$^-$ *lac*Δ*X74 his trp tsx ton rpsE*], JC3051 [F$^-$ *lac*Δ*X74 his trp rpsL tsx ton* (λ)], and SE5000 [F$^-$ *araD139 lac*Δ*U169 rpsL relA thi recA56*] are described elsewhere (31). Strain XK100 is a spontaneous spectinomycin-resistant derivative of BL21(DE3), an *E. coli* B derivative that carries a chromosomal T7 RNA polymerase gene controlled by the *lacUV5* promoter (35). Strain CC118 is F$^-$ *lac*Δ*X74 araD139* Δ(*ara-leu*)*7697 phoA20 galE galK thi rspE rpoB argE*(Am) *recA1* (24).

DNA cloning and sequencing. Cloning and restriction enzyme analysis of DNA fragments were performed by stan-

TABLE 1. Plasmids

Plasmid	*tra* fragment size (kb) and ends[a]	Vector and vector junction sites	Construction, source, or reference
pKI159	2.6 *Eco*RV	pACYC177 *amp* *Hin*cII	Maneewannakul et al. (23)
pKI175	6.2 *Ava*I	pACYC177 *kan* *Xma*I	Wu et al. (41)
pKI184	4.1 *Hin*cII	pACYC177 *amp* *Hin*cII	Wu et al. (41)
pKI188	1.5 *Hin*cII	pACYC177 *amp* *Hin*cII	*Eco*RV deletion of pKI184 (this paper)
pKI272	3.8 *Eco*RV	pBR322 *tcy* *Eco*RV	Maneewannakul et al. (23)
pKI338	1.6 *Eco*RV-*Sca*I	pUC18 *Hin*cII	This paper
pKI343	1.6 *Eco*RV-*Sca*I + *trbC343*	pUC18 *Hin*cII	*Bsm*I-S1 nuclease digest of pKI338 ligated with the pUC4-KISS *Pst*I-S1 nuclease Km^r fragment[b] (this paper)
pKI372	See Fig. 1 and 4		Derived from pKI375 by 3′ exonuclease III digestion (this paper)
pKI373	See Fig. 1		Derived from pKI375 by 3′ exonuclease III digestion (this paper)
pKI375	3.0 *Xmn*I	pBS/KS+ *lacZ*α *Eco*RV	This paper
pKI451	1.62 *Eco*RV-*Sca*I	pKI497 *amp* *Sca*I	This paper
pKI460	1.62 *Eco*RV-*Sca*I + *trbC460*	pKI497 *amp* *Sca*I	*Pvu*II digest of pKI451 ligated with the pUC4K *Bam*HI Km^r fragment (this paper)
pOX38	45.4 *Hin*dIII	None	Circularized F fragment (Guyer et al. [8])
pOX38-Km	45.4 *Hin*dIII	None	Tn*5* *kan* *Hin*dIII fragment in the pOX38 *Hin*dIII site (Chandler and Galas [3])
pOX38*trbC343*	45.4 *Hin*dIII + *trbC343*	None	In vivo recombination of pKI343 and pOX38
pOX38*trbC460*	45.4 *Hin*dIII + *trbC460*	None	In vivo recombination of pKI460 and pOX38

[a] Except as noted, transcription from the vector *amp* promoter (or *kan* and *tcy* promoters of pKI175 and pKI272, respectively) proceeds into the *tra* DNA inserts in the *tra* operon direction (from left to right in Fig. 1).
[b] The orientation of the Km^r gene insert is opposite of that of *trbC* (Fig. 1).

dard procedures (41). Exonuclease III deletion was performed as described by Henikoff (12), except that either a nonsense codon linker (linker number 1062; New England BioLabs, Inc., Beverly, Mass.) containing stop codons in all three reading frames (for pKI372 and pKI373) or a *phoA* DNA fragment from pS2 (for pN15) was added prior to ligation. Nucleotide sequence determinations were performed on M13mp8 and M13mp9 clones with the Sequenase system (United States Biochemical Corp.) and synthetic oligonucleotide primers purchased from the Advanced DNA Technology Laboratory (Department of Biology, Texas A&M University). Both dGTP and dITP reaction mixtures were analyzed, and both DNA strands were sequenced. The Genetics Computer Group Sequence Analysis Software Package Version 6.2 (5) was used for computer analyses.

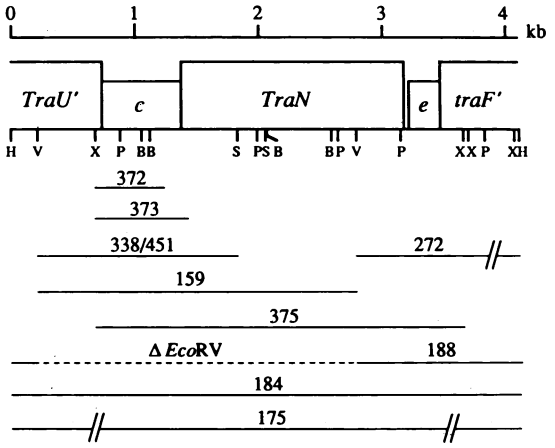

FIG. 1. Map of F transfer region genes within the 4.1-kb *Hin*cII fragment carried by pKI184. At the top of the map, the numbers indicate DNA lengths in kilobase pairs (kb), and boxes indicate the positions and sizes of gene sequences. Genes *trbC* and *trbE* are indicated by *c* and *e*, respectively; a prime indicates that only part of the gene sequence is present. The positions of *Bsm*I (B), *Eco*RV (V), *Hin*cII (H), *Sca*I (S), *Pvu*II (P), and *Xmn*I (X) restriction sites are shown. The lines below the map indicate the DNA sequences carried by various pKI plasmids; a broken line indicates the deletion in pKI188, and slashes indicate that the cloned fragment extends beyond the *tra* region shown. The map is based on DNA sequences determined for *traU* (29), *trbC* (this paper), *traN* (22), and *traF* (40).

Complementation experiments. For determining the frequency of conjugative transfer in bacterial matings, donors and recipients were grown at 37°C in LB medium (28) to a density corresponding to approximately 2×10^8 cells per ml. A mixture of 0.1 ml of donors and 0.4 ml of recipients was mated for 45 min at 37°C prior to plating of dilutions on selective plates. Phage sensitivities were determined by spot tests or plaque counts as described by Moore et al. (29).

Detection and localization of plasmid protein products. For maxicell analysis of protein expression, plasmids were introduced into strain SE5000 by transformation and maxicell cultures were prepared and labeled with [^{35}S]methionine as described previously (31). When indicated, ethanol (final concentration, 9%) was added prior to the addition of the radioactive label. The products of pKI372, pKI373, and pKI375 and other pBS/KS+ derivatives were labeled in the host, XK100. In these experiments, T7 RNA polymerase was induced by the addition of 1 mM isopropyl-β-D-thiogalactopyranoside; 30 to 60 min later, rifampin (final concentration, 100 μg/ml) was added and incubation was continued for 60 min prior to labeling for 5 min with [^{35}S]methionine. Procedures for analysis of samples by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and autoradiography were as described elsewhere (15, 29–31).

Gels were poured with an exponential gradient of 10 to 16% acrylamide and run in a Bio-Rad Protean II gel apparatus in a cooled chamber at a constant power of 10 W.

Periplasmic protein fractions were prepared from labeled maxicell cultures by three different protocols based on methods described by others. For obtaining a chloroform shock periplasmic fraction (2), 1 to 1.5 ml of a [^{35}S]methionine-labeled maxicell culture was spun for 3 min in an Eppendorf centrifuge, and the cell pellet was vortexed with 30 μl of chloroform and incubated at room temperature for 15 min. After the addition of 200 μl of 0.01 M Tris-HCl (pH 8.0) buffer and a 15-min spin in a microcentrifuge, 100 to 150 μl of the supernatant was retained for analysis. For obtaining an osmotic shock periplasmic fraction (11, 25), 1 to 1.5 ml of a [^{35}S]methionine-labeled maxicell culture was spun for 3 min in an Eppendorf centrifuge, and the cell pellet was washed twice with 0.01 M Tris-HCl (pH 8.0)–0.3 M NaCl buffer and suspended in 150 μl of preshock buffer (100 mM Tris-HCl [pH 8.0], 0.5 mM EDTA, 20% sucrose, 1 mM phenylmethylsulfonyl fluoride). After 20 min of incubation at room temperature, the mixture was centrifuged for 1 to 2 min, and the cell pellet was suspended in 200 μl of ice-cold 0.5 mM $MgCl_2$ and placed on ice for 10 min. Finally, the mixture was spun for 15 min in a microcentrifuge, and 100 to 150 μl of the supernatant was retained for analysis. Fractionation of periplasmic proteins by polymyxin B treatment was performed as described by Hirst and Holmgren (13).

Nucleotide sequence accession number. The GenBank/EMBL nucleotide sequence accession number for the *trbC* sequence reported in this paper is M60427.

RESULTS

Wu et al. (41) had previously observed that plasmids pKI175 and pKI184 both expressed two polypeptides with apparent molecular weights (M_a) of 23,500 and 21,500. To locate the region encoding these polypeptides more precisely, we constructed various additional plasmids carrying sequences included in the *Hin*cII *tra* DNA fragment expressed by pKI184 (Fig. 1). Analysis of the labeled products that these plasmids expressed in maxicells showed that pKI338 and other constructs (pKI159 and pKI375) including the same 1.6-kb *Eco*RV-*Sca*I fragment (Fig. 1) expressed both the 23.5- and 21.5-kDa polypeptides (Fig. 2). Plasmids such as pKI188 and pKI272 did not express either polypeptide product (21). Subsequently, we also examined two derivatives made by 3′ exonuclease III deletion of the *Xmn*I *tra* fragment in pKI375. Plasmid pKI373 still expressed both the 23.5- and 21.5-kDa polypeptides, while pKI372 expressed two truncated polypeptides (Fig. 2 and see below).

***trbC* products.** To test whether the 23.5- and 21.5-kDa polypeptides represented the precursor and the processed product of a single gene, we examined the effect of ethanol on the expression of these proteins. When ethanol was present during the labeling period, the 23.5-kDa product expressed by plasmids pKI175 and pKI338 accumulated and the 21.5-kDa product was not detected (Fig. 3A, lane 2, and 3B, lane 1). However, after maxicells labeled in the presence of ethanol were washed twice, resuspended in medium containing an excess of unlabeled methionine, and incubated for a further 30 min, the 23.5-kDa band diminished in intensity and the 21.5-kDa band appeared (Fig. 3A, lane 3, and 3B, lane 2). We concluded that the 21.5-kDa polypeptide was indeed derived from the 23.5-kDa precursor. Both of these products therefore appeared to be encoded by a previously unidentified F transfer region gene located between *traU* and *traN*. We named this gene *trbC*.

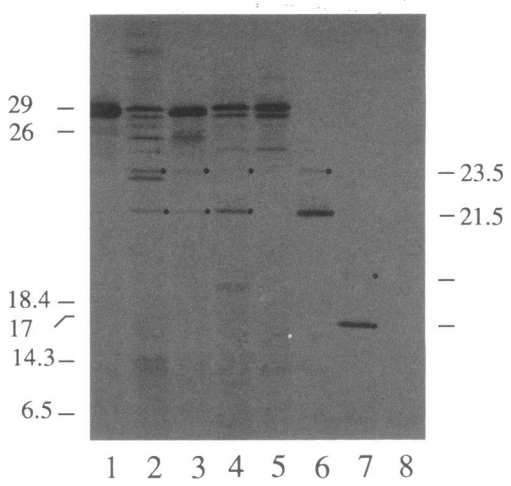

FIG. 2. Expression of the 23.5- and 21.5-kDa polypeptides from various plasmids. Plasmid products were labeled with [^{35}S]methionine in maxicells or in whole cells after the induction of T7 polymerase and the addition of rifampin. A portion of the autoradiogram of an SDS-PAGE gel is shown. Lane numbers correspond to samples carrying pACYC177 (1), pKI175 (2), pKI159 (3), pKI338 (4), pUC18 (5), pKI373 (6), pKI372 (7), and pBS/KS+ (8). Numbers at the left indicate the positions and sizes (in kilodaltons) of molecular mass markers; dots in lanes and lines at the right mark the positions of the 23.5- and 21.5-kDa products and of the truncated products expressed by pKI372.

***trbC* sequence.** We determined the nucleotide sequence of the 1.621-kb *Eco*RV-*Sca*I fragment carried by plasmid pKI338. The portion of this sequence that contains *trbC* is shown in Fig. 4. The complete sequences of the *traU* and *traN* genes are reported elsewhere (22, 29; GenBank/EMBL accession number M34695).

As indicated in Fig. 4, a single open reading frame was found to span the region between *traU* and *traN*. This

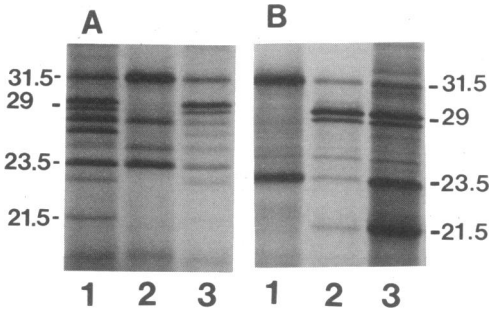

FIG. 3. Effect of ethanol on *trbC* polypeptide products. (A) Proteins expressed by pKI175 in maxicells were labeled with [^{35}S]methionine for 15 min with no ethanol present (lane 1) or with 9% ethanol present (lane 2) or for 15 min with 9% ethanol followed by a 30-min chase with unlabeled methionine after the removal of ethanol (lane 3). Numbers at the sides indicate the sizes (in kilodaltons) and positions of TrbC (21.5 kDa), TrbC precursor (23.5 kDa), β-lactamase (29 kDa), and β-lactamase precursor (31.5 kDa). Only a portion of the autoradiogram of the SDS-PAGE gel is shown. (B) As in panel A, except that maxicells carried pKI338. Samples were labeled without ethanol (lane 3), with ethanol (lane 1), or with ethanol followed by a 30-min chase (lane 2).

FIG. 4. Nucleotide sequence of *trbC*. The 212-amino-acid translation product is shown, as are amino acids at the C terminus of the *TraU* product and the N terminus of the *TraN* product. Potential ribosome binding site sequences and the putative *trbC* product membrane signal sequence are underlined. The positions of the *Bsm*I, *Hae*II, *Nae*I, *Pst*I, and *Pvu*II restriction sites are indicated. Nucleotide 1 of the sequence is nucleotide 1021 of the *traU* sequence of Moore et al. (29). The *tra* segment carried by pKI372 ends at nucleotide 548, as marked.

reading frame begins with two methionine codons. We suggest that translation starts at the second AUG, 5 nucleotides after the putative GAGGT ribosome binding site, since this placement fits the proposition that spacing between the end of a ribosome binding site and a translation start site is 7 ± 2 nucleotides (34). However, the possibility that translation starts at the first AUG or that both AUGs act as initiation codons has not been excluded, since there is evidence that spacing can be only 2 nucleotides (7). The *trbC* sequence shown in Fig. 4 encodes a 212-amino-acid M_r-23,432 polypeptide that includes a canonical 21-amino-acid amino-terminal membrane signal sequence. Removal of the signal sequence would result in a 191-amino-acid M_r-21,225 polypeptide. These sizes are in very close agreement with the sizes of the *trbC* products that we observed. Furthermore, the DNA sequence of pKI372 showed that the C-terminal region of the *trbC* open reading frame had been deleted in this derivative (Fig. 4). The truncated *trbC* products expected from the sequence would be 19,524 and 17,317 Da, sizes again correlating well with the sizes of the pKI372 products that we observed. The DNA sequence of pKI373 included the entire *trbC* open reading frame and the coding sequence for the first 20 amino acids of the *traN* product.

Further evidence that the *trbC* product includes a signal peptide transport sequence was obtained by the construction of fusions with *phoA*. Figure 5 shows the N-terminal structure of one interesting *trbC*::*phoA* fusion clone (pN15) obtained following exonuclease III deletion and *phoA* cassette insertion. The nucleotide sequence of pN15 was found to encode only the first 14 amino acids of the *trbC* product signal peptide fused to the sixth amino acid of the mature alkaline phosphatase protein. The fusion protein signal peptide still carried two N-terminal positive charges and a reasonable hydrophobic core (seven amino acids). The introduction of pN15 into a *phoA* strain (CC118) resulted in the strong expression of alkaline phosphatase activity, as indicated on plates containing 5-bromo-4-chloro-3-indolyl-phosphate (final concentration, 40 μg/ml). Since alkaline phosphatase activity is known to be expressed only when the protein is located in the periplasm (27), this portion of the TrbC signal peptide apparently suffices for the transport of alkaline phosphatase across the cytoplasmic membrane.

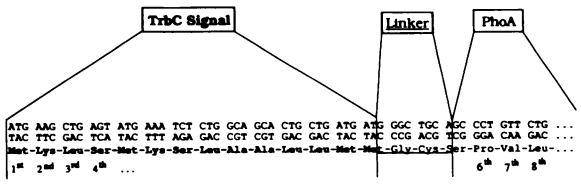

FIG. 5. DNA sequence of the pN15 *trbC*::*phoA* fusion region and the protein sequence it encodes. Numbers refer to amino acid residues in the *trbC* product signal sequence and in the sequence of the mature PhoA protein. The linker sequence stems from the multicloning site in pS2.

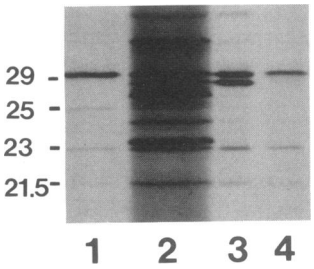

FIG. 6. Cellular location of TrbC. Plasmid products were labeled with [^{35}S]methionine in maxicells carrying pKI175, fractionated, and analyzed by SDS-PAGE. A portion of the autoradiogram is shown. Numbers at the left indicate the sizes (in kilodaltons) of periplasmic proteins in fractions obtained by polymyxin B treatment (lane 1), osmotic shock (lane 3), and chloroform shock (lane 4) and correspond to β-lactamase (29 kDa), TraF (25 kDa), TraW (23 kDa), and TrbC (21.5 kDa). An unfractionated pKI175 maxicell sample is shown in lane 2.

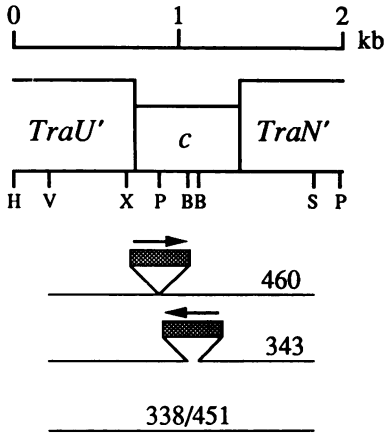

FIG. 7. Construction of *trbC* insertion mutations. The map of the *trbC* region is drawn as in Fig. 1. Below the map the numbered lines indicate the DNA present in plasmids pKI338, pKI451, pKI460, and pKI343. Hatched lines and arrows show the positions and orientations of the Km^r gene inserts (1.25 kb; not drawn to scale).

Localization of the *trbC* product. The sequence of TrbC suggested that it is primarily a hydrophilic protein. Furthermore, in a previous report, Wu et al. (40) observed that the 21.5-kDa product of pKI175 was enriched in a periplasmic protein fraction prepared from maxicell samples. To test this finding, we fractionated labeled maxicells by several different procedures. As shown in Fig. 6, the 21.5-kDa mature TrbC protein expressed by pKI175 was present in periplasmic fractions obtained by polymyxin B treatment (lane 1), osmotic shock (lane 3), or chloroform shock (lane 4). These fractions also contained β-lactamase (29 kDa) and additional pKI175 products identified elsewhere as periplasmic proteins TraU (33 kDa [29]), TraF (25 kDa [40]), and TraW (23 kDa [20, 23]). They did not contain proteins expected to remain in other fractions. For example, the unprocessed polypeptide precursors of the periplasmic proteins were prominent bands in membrane fractions or unfractionated cell samples (lane 2) but were undetectable in our periplasmic fractions.

Construction of *trbC* mutant derivatives. To test whether the expression of *trbC* was essential to the F plasmid conjugative transfer system, we constructed Km^r resistance gene insertion mutations in *trbC* in vitro and then crossed these mutations in vivo onto pOX38. Plasmid pOX38 was originally derived from F by recircularization of the large F *Hin*dIII fragment which includes the F transfer and repFIA replication regions but not Tn*1000* or insertion sequence elements (8). The technique that we used has been successfully used for the analysis of other *tra* region gene functions (17, 29).

Two plasmids carrying Km^r gene insertion mutations in *trbC* were constructed in vitro (Fig. 7). To obtain plasmid pKI343, we digested pKI338 DNA with *Bsm*I to remove a 152-bp segment from *trbC*. The larger pKI338 fragment was treated with S1 nuclease and ligated with an S1 nuclease-digested pUC4-KISS *Pst*I fragment carrying the Tn*903* Km^r gene. Restriction fragment analysis of purified pKI343 DNA confirmed that the *Bsm*I fragment within *trbC* had been deleted and showed that, in this construction (mutation *trbC343*), the orientation of the Km^r gene insert was opposite to that of the *tra* genes (Fig. 7). Analysis of plasmid products in maxicells confirmed that plasmid pKI343 did not express the 21.5- or 23.5-kDa *trbC* products expressed by the parental plasmid, pKI338 (21). The second insertion mutant plasmid, pKI460, was constructed by cutting pKI451 at the unique *Pvu*II site in *trbC* and inserting the end-filled *Bam*HI Km^r gene fragment from pUC4K at this position. Restriction enzyme analysis showed that, in this construction (mutation *trbC460*), the Km^r gene was inserted at the *Pvu*II site and oriented in the *tra* direction (Fig. 7).

Derivatives of plasmid pOX38 carrying each of these *trbC* Km^r insertion mutations were constructed by an in vivo recombination procedure detailed elsewhere (17, 29). For obtaining pOX38*trbC343*, plasmid pKI343 (Amp^r Km^r) was transformed into a pOX38 donor strain, and the transformant was grown, mixed with a culture of the Nal^r strain XK1200, and incubated overnight. Km^r Nal^r transconjugants were selected, and Amp^s transconjugants were identified. In spot tests with the recipient strain, XK3051, all of these Km^r Nal^r Amp^s transconjugants were transfer deficient. Comparison of *Eco*RV and *Hin*cII restriction digests of pOX38 DNA with digests of plasmid DNA purified from one of the Amp^s transconjugants confirmed that it was a recombinant carrying the expected Km^r gene insert in the pOX38 *trbC* gene. This plasmid was named pOX38*trbC343*. A pOX38 derivative carrying the *trbC460* mutation was obtained in a similar manner. In this case, after the introduction of pKI460 (Tet^r Km^r) into the pOX38 donor strain, Nal^r Km^r Tet^s transconjugants of XK1200 were identified. All of these Nal^r Km^r Tet^s transconjugants were transfer deficient; after purification and restriction enzyme analysis to confirm its structure, the plasmid in a representative transconjugant was named pOX38*trbC460*.

Analysis of pOX38 *trbC* mutant derivatives. Strains carrying pOX38*trbC343* and pOX38*trbC460* were tested for sensitivity to F-pilus-specific phages and the capacity to transfer Km^r to recipients. For comparison, a strain carrying pOX38-Km, a transmissible derivative carrying a Km^r gene insertion in a position outside the *tra* region (3), was used as a wild-type control. The results are summarized in Table 2. The transfer frequencies of both pOX38*trbC343* and pOX38 *trbC460* were drastically reduced in comparison with that of pOX38-Km. In addition, strains carrying either *trbC* mutant plasmid appeared completely resistant to F-pilus-specific RNA phages. Although a slight sensitivity to F-pilus-specific DNA phages was observed in spot tests, the filamentous

TABLE 2. Analysis of pOX38 *trbC* mutants

Plasmid(s) present	No. of transconjugants/ 100 donors[a]	Sensitivity[b] to phage:	
		f1, M13, and fd	Qβ, f2, and R17
pOX38-Km[c] (wild type)	82.5	S	S
pOX38*trbC343*	$<1 \times 10^{-5}$	(R)	R
pOX38*trbC343* + pKI175	133.0[d]	S	S
pOX38*trbC343* + pKI451[e]	36.6[d]	S	S
pOX38*trbC460*	2×10^{-5}	(R)	R
pOX38*trbC460* + pKI175	118.0[d]	S	S
pOX38*trbC460* + pKI451[e]	31.6[d]	S	S

[a] Donors were XK1200 derivatives; Km^r Sm^r transconjugants of the recipient, JC3051, were selected.

[b] S, plating efficiency comparable to that of the F*lac* control; R, no phage sensitivity detected; (R), plaques were not seen, but limited lysis was detectable in spot tests.

[c] pOX38-Km contains the normal F *tra* operon sequence. In this plasmid, a *kan* gene inserted into the pOX38 *Hin*dIII site provides a selective marker (3).

[d] All transconjugants tested were transfer deficient and resistant to F-pilus-specific phages.

[e] Donor cultures carrying pKI451 grew poorly.

DNA phages were also unable to form plaques on the *trbC* mutant strains.

To test whether a TrbC$^+$ plasmid could complement the defects associated with the *trbC343* and *trbC460* mutations, we also tested the transfer proficiency and phage sensitivity of derivatives containing pKI451 or pKI175. The introduction of either plasmid into the pOX38 *trbC* mutant strains resulted in the restoration of both transfer proficiency and F-pilus-specific phage sensitivity (Table 2). Since *trbC* is the only *tra* gene expressed by pKI451, these results unequivocally demonstrated that both transfer proficiency and F-pilus-specific phage sensitivity are dependent on TrbC.

DISCUSSION

The expression of the 21.5- and 23.5-kDa products that we characterized was first observed during analyses of proteins expressed by various transducing phages and plasmids carrying segments of F plasmid DNA (15, 19, 41). Since the processing of *tra* operon products was originally thought to be unusual (1), a 1986 map of the F transfer region was drawn to accommodate two loci (*trbC* and *trbD*) to account for the production of both polypeptides (16). However, the data presented here demonstrate that the two polypeptides stem from a single F *tra* operon gene, *trbC*, located between the *traU* and *traN* genes. As indicated in Fig. 4, translation of *trbC* is expected to be initiated 6 to 9 nucleotides distal to the *traU* stop codon, while the initiation codon for the translation of *traN* actually overlaps the C-terminal codon for *trbC*. Plasmids carrying the *trbC* region were found to express a 23.5-kDa precursor polypeptide that accumulated when ethanol was present and was processed to the 21.5-kDa protein when ethanol was removed. In close agreement with these data, the DNA sequence that we determined for *trbC* encodes a 212-amino-acid M_r-23,432 polypeptide which includes a characteristic amino-terminal signal sequence; the removal of a 21-amino-acid signal peptide results in a 191-amino-acid M_r-21,225 polypeptide, the TrbC protein. We found mature TrbC to be present in periplasmic fractions prepared by several different procedures. The sequence of TrbC presented in Fig. 4 is also consistent with this finding. In a hydropathy plot (6, 18), mature TrbC appears to be mostly hydrophilic. However, there is a short hydrophobic segment at the C terminus.

Our data also demonstrate that *trbC* gene function is essential to the F plasmid conjugative transfer system. We introduced two different Km^r gene insertion mutations into the *trbC* gene of the transmissible F plasmid derivative pOX38. The transfer frequencies of the pOX38 *trbC* mutant plasmids were reduced to approximately 10^{-7} wild-type transfer frequencies. Cells carrying the mutant plasmids were also found to be resistant to infection by F-pilus-specific phages. Both transfer proficiency and F-pilus-specific phage sensitivity were restored when plasmid pKI451 was introduced into the pOX38 *trbC* mutant strains. As *trbC* is the only *tra* region gene expressed by pKI451, we can conclude that both the phage resistance and the transfer deficiency phenotypes caused by our *trbC* mutations were due to the loss of *trbC* function. Thus, although *trbC* is part of the *tra* operon, neither an upstream- nor a downstream-oriented Km^r gene insertion appeared to markedly affect the expression of neighboring genes, such as *traU* and *traN*, which are also required for plasmid transfer. Apparently, transcription of the *tra* operon, presumably from the $P_{Y\text{-}X}$ *tra* operon promoter, is not severely impeded by the Km^r gene insertions and the expression of promoter-distal genes remains at levels sufficient for transfer.

The resistance to F-pilus-specific phages observed to result from *trbC* mutations suggests that TrbC product function is required for the expression of F pili. Additional studies have shown that F-pilin subunits can be detected in membrane preparations from *trbC* mutants (10), while a preliminary electron microscope examination has indicated that pOX38 *trbC* mutant strains either do not express F pili, or express the filaments very rarely relative to pOX38 strains (21). Thus, *trbC* appears to be one of the large group of *tra* operon genes that participate in the assembly of F-pilus filaments. Like TrbC, several of these *tra* operon products have also been shown to be periplasmic proteins, and it seems probable that these F plasmid products function together in a protein complex (9, 29, 39, 40).

It is worth noting that, unlike strains bearing mutations in most F-pilus assembly genes, lawns of pOX38 *trbC* mutant strains exhibited a slight sensitivity to F-pilus-specific filamentous DNA phages in spot tests. This result suggests that further study of *trbC* mutant strains might be useful in identifying an intermediate stage in F-pilus assembly. Although our current data do not rule out the possibility that a small proportion of the cells in pOX38 *trbC* mutant cultures produce pili, an alternative explanation is that the slight sensitivity to filamentous phages reflects the availability of unextended F-pilus tips on the cell surface. The latter hypothesis has been invoked to explain the similar phenotype of strains carrying *traC1044*, an unusual temperature-sensitive mutation resulting from an amino acid substitution near the C terminus of the 99,066-Da TraC protein (32, 33).

ACKNOWLEDGMENTS

This work was supported by Public Health Service grant AI14426 from the National Institutes of Health.

We thank Y. Chang and M. Benedik for their courtesy in sharing their strain and plasmid collections.

REFERENCES

1. **Achtman, M., P. A. Manning, C. Edelbluth, and P. Herrlich.** 1979. Export without proteolytic processing of inner and outer membrane proteins encoded by F sex factor *tra* cistrons in *Escherichia coli* minicells. Proc. Natl. Acad. Sci. USA **76**:4837–4841.

2. **Ames, G. F.-L., C. Prody, and S. Kustu.** 1984. Simple, rapid, and quantitative release of periplasmic proteins by chloroform. J. Bacteriol. **160:**1181–1183.
3. **Chandler, M., and D. Galas.** 1983. Cointegrate formation mediated by Tn9. II. Activity of IS1 is modulated by external DNA sequences. J. Mol. Biol. **170:**61–91.
4. **Chang, A. C. Y., and S. N. Cohen.** 1978. Construction and characterization of amplifiable multicopy DNA cloning vehicles derived from the p15A cryptic miniplasmid. J. Bacteriol. **134:** 1141–1156.
5. **Devereux, J., P. Haeberli, and O. Smithies.** 1984. A comprehensive set of sequence analysis programs for the VAX. Nucleic Acids Res. **12:**387–395.
6. **Engelman, D. M., T. A. Steitz, and A. Goldman.** 1986. Identifying nonpolar transbilayer helices in amino acid sequences of membrane proteins. Annu. Rev. Biophys. Biophys. Chem. **15:**321–353.
7. **Gold, L., and G. Stormo.** 1987. Translation initiation, p. 1302–1307. *In* F. C. Neidhardt, J. L. Ingraham, K. B. Low, B. Magasanik, M. Schaechter, and H. E. Umbarger (ed.), *Escherichia coli* and *Salmonella typhimurium*: cellular and molecular biology. American Society for Microbiology, Washington, D.C.
8. **Guyer, M. S., R. R. Reed, J. A. Steitz, and K. B. Low.** 1980. Identification of a sex-factor affinity site in *E. coli* as γδ. Cold Spring Harbor Symp. Quant. Biol. **45:**135–140.
9. **Ham, L. M., N. Firth, and R. Skurray.** 1989. Nucleotide sequence of the F plasmid transfer gene, *traH*: identification of a new gene and a promoter within the transfer operon. Gene **75:**157–165.
10. **Hamilton, C. M.** Unpublished data.
11. **Hazelbauer, G. L., and S. Harayama.** 1979. Mutants in transmission of chemotactic signals from two independent receptors of *E. coli*. Cell **16:**617–625.
12. **Henikoff, S.** 1987. Unidirectional digestion with exonuclease III in DNA sequence analysis. Methods Enzymol. **155:**156–165.
13. **Hirst, T. R., and J. Holmgren.** 1987. Transient entry of enterotoxin subunits into the periplasm occurs during their secretion from *Vibrio cholerae*. J. Bacteriol. **169:**1037–1045.
14. **Hoffman, C. S., and A. Wright.** 1985. Fusion of secreted proteins to alkaline phosphatase: an approach for studying protein secretion. Proc. Natl. Acad. Sci. USA **82:**5107–5111.
15. **Ippen-Ihler, K., D. Moore, S. Laine, D. A. Johnson, and N. Willetts.** 1984. Synthesis of F-pilin polypeptide in the absence of F *traJ* product. Plasmid **11:**116–129.
16. **Ippen-Ihler, K. A., and E. G. Minkley, Jr.** 1986. The conjugation system of F, the fertility factor of *Escherichia coli*. Annu. Rev. Genet. **20:**593–624.
17. **Kathir, P., and K. Ippen-Ihler.** Construction and characterization of derivatives carrying insertion mutations in F plasmid transfer region genes, *trbA*, *artA*, *traQ*, and *trbB*. Plasmid, in press.
18. **Kyte, J., and R. F. Doolittle.** 1982. A simple method for displaying the hydropathic character of a protein. J. Mol. Biol. **157:**105–132.
19. **Laine, S., D. Moore, P. Kathir, and K. Ippen-Ihler.** 1985. Genes and gene products involved in the synthesis of F-pili, p. 535–553. *In* D. R. Helinski, S. N. Cohen, D. B. Clewell, D. A. Jackson, and A. Hollaender (ed.), Plasmids in bacteria. Plenum Publishing Corp., New York.
20. **Maneewannakul, S.** 1990. Ph.D. thesis. Texas A&M University, College Station.
21. **Maneewannakul, S.** Unpublished data.
22. **Maneewannakul, S., P. Kathir, and K. Ippen-Ihler.** Unpublished data.
23. **Maneewannakul, S., P. Kathir, D. Moore, L.-A. Le, J. H. Wu, and K. Ippen-Ihler.** 1987. Location of F plasmid transfer operon genes *traC* and *traW* and identification of the *traW* product. J. Bacteriol. **169:**5119–5124.
24. **Manoil, C., and J. Beckwith.** 1985. Tn*phoA*: a transposon probe for protein export signals. Proc. Natl. Acad. Sci. USA **82:**8129–8133.
25. **Manoil, C., and J. Beckwith.** 1986. A genetic approach to analyzing membrane protein topology. Science **233:**1403–1408.
26. **Mead, D. A., and B. Kemper.** 1988. Chimeric single-stranded DNA phage-plasmid cloning vectors, p. 85–102. *In* R. L. Rodriguez and D. T. Denhardt (ed.), Vectors: a survey of molecular cloning vectors and their uses. Butterworth Publishers, Boston.
27. **Michaelis, S., H. Inouye, D. Oliver, and J. Beckwith.** 1983. Mutations that alter the signal sequence of alkaline phosphatase in *Escherichia coli*. J. Bacteriol. **154:**366–374.
28. **Miller, J. H.** 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
29. **Moore, D., K. Maneewannakul, S. Maneewannakul, J. Wu, K. Ippen-Ihler, and D. E. Bradley.** 1990. Characterization of the F plasmid conjugative transfer gene *traU*. J. Bacteriol. **172:**4263–4270.
30. **Moore, D., B. A. Sowa, and K. Ippen-Ihler.** 1982. A new activity in the F *tra* operon which is required for F-pilin synthesis. Mol. Gen. Genet. **188:**459–464.
31. **Moore, D., J. H. Wu, P. Kathir, C. M. Hamilton, and K. Ippen-Ihler.** 1987. Analysis of transfer genes and gene products within the *traB-traC* region of the *Escherichia coli* fertility factor, F. J. Bacteriol. **169:**3994–4002.
32. **Schandel, K. A., S. Maneewannakul, K. Ippen-Ihler, and R. E. Webster.** 1987. A *traC* mutant that retains sensitivity to f1 bacteriophage but lacks F pili. J. Bacteriol. **169:**3151–3159.
33. **Schandel, K. A., S. Maneewannakul, R. A. Vonder Haar, K. Ippen-Ihler, and R. E. Webster.** 1990. Nucleotide sequence of the F plasmid gene *traC* and identification of its product. Gene **96:**137–140.
34. **Stormo, G. D.** 1986. Translation initiation, p. 195–224. *In* W. Reznikoff and L. Gold (ed.), Maximizing gene expression. Butterworths, Stoneham, Mass.
35. **Studier, F. W., and B. A. Moffatt.** 1986. Use of bacteriophage T7 RNA polymerase to direct selective high-level expression of cloned genes. J. Mol. Biol. **189:**113–130.
36. **Sutcliffe, J. G.** 1978. Nucleotide sequence of *Escherichia coli* plasmid pBR322. Proc. Natl. Acad. Sci. USA **75:**3737–3741.
37. **Tsai, M.-M., R. Y.-P. Wong, A. Hoang, and R. Deonier.** 1987. Transposition of Tn*1000*: in vivo properties. J. Bacteriol. **169:** 5556–5562.
38. **Willetts, N., and R. Skurray.** 1987. Structure and function of the F factor and mechanism of conjugation, p. 1110–1133. *In* F. C. Neidhardt, J. L. Ingraham, K. B. Low, B. Magasanik, M. Schaechter, and H. E. Umbarger (ed.), *Escherichia coli* and *Salmonella typhimurium*: cellular and molecular biology. American Society for Microbiology, Washington, D.C.
39. **Wu, J. H., and K. Ippen-Ihler.** 1989. Nucleotide sequence of *traQ* and adjacent loci in the *Escherichia coli* K-12 F-plasmid transfer operon. J. Bacteriol. **171:**213–221.
40. **Wu, J. H., P. Kathir, and K. Ippen-Ihler.** 1988. The product of the F plasmid transfer operon gene, *traF*, is a periplasmic protein. J. Bacteriol. **170:**3633–3639.
41. **Wu, J. H., D. Moore, T. Lee, and K. Ippen-Ihler.** 1987. Analysis of *E. coli* K12 F factor transfer genes: *traQ*, *trbA* and *trbB*. Plasmid **18:**54–69.