

## Characterization, Localization, and Sequence of F Transfer Region Products: the Pilus Assembly Gene Product TraW and a New Product, TrbI

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Received 23 April 1992/Accepted 29 June 1992

The *traW* gene of the *Escherichia coli* K-12 sex factor, F, encodes one of the numerous proteins required for conjugative transfer of this plasmid. We have found that the nucleotide sequence of *traW* encodes a 210-amino-acid, 23,610-Da polypeptide with a characteristic amino-terminal signal peptide sequence; in DNA from the *F lac traW546* amber mutant, the *traW* open reading frame is interrupted at codon 141. Studies of *traW* expression in maxicells in the presence and absence of ethanol demonstrate that the *traW* product does undergo signal sequence processing. Cell fractionation experiments additionally demonstrated that mature TraW is a periplasmic protein. Electron microscopy also showed that *F lac traW546* hosts do not express F pili, confirming that TraW is required for F-pilus assembly. Our nucleotide sequence also revealed the existence of an additional gene, *trbI*, located between *traC* and *traW*. The *trbI* gene encodes a 128-amino-acid polypeptide which could be identified as a 14-kDa protein product. Fractionation experiments demonstrated that TrbI is an intrinsic inner-membrane protein. Hosts carrying the pOX38-*trbI::kan* insertion mutant plasmids that we constructed remained quite transfer proficient but exhibited increased resistance to F-pilus-specific phages. Mutant plasmids pOX38-*trbI472* and pOX38-*trbI473* expressed very long F pili, suggestive of a pilus retraction deficiency. Expression of an excess of TrbI in hosts carrying a wild-type pOX38 plasmid also caused F-pilus-specific phage resistance. The possibility that TrbI influences the kinetics of pilus outgrowth and/or retraction is discussed.

The transfer (*tra*) region of the *Escherichia coli* K-12 plasmid F contains the numerous genes required for conjugative transfer of the plasmid. A large subset of these, including *traW*, is involved in expression of F pili. These long filaments extend from the surface of F donor bacteria and initiate mating contacts with recipient cells (for reviews, see references 8, 9, and 26). The single *traW* mutant available, the transfer-deficient amber mutant plasmid *F lac traW546*, was isolated by Miki et al. (17), who found that it could complement transfer of plasmids carrying defects in all other known *tra* cistrons. Subsequent studies located *traW* between the transfer region genes *traC* and *traU* and identified a polypeptide with an apparent molecular weight ( $M_a$ ) of 23,000 as the *traW* gene product (14). Recently, the nucleotide sequences of both *traC* and *traU* have been reported (18, 23). We now report the sequence of the intervening *tra* region, which includes a previously uncharacterized locus, *trbI*, as well as the *traW* gene. Our studies show that the *traW* product is subject to signal sequence processing and translocation to the periplasmic space, while the *trbI* product is an intrinsic inner membrane protein that can affect F-pilus-specific phage infection and F-pilus length.

### MATERIALS AND METHODS

**Bacterial strains and plasmids.** The construction and properties of plasmids expressing F *tra* region genes are summarized in Table 1. The *tra* region segments included on these plasmids are also indicated in Fig. 1. Vectors used were

pACYC177 (4), pLa2311 (21), and pKI497 (15). The latter is a derivative of pTZ18U (16) which carries *tcy* in place of *lacZ $\alpha$* . The Tn903 kanamycin resistance (*kan*) gene cassette used came from plasmid pUC4K (Pharmacia Inc., Molecular Biology Division, Piscataway, N.J.). Matings and selections used for in vivo construction of pOX38 mutant derivatives were as previously described (11, 15). pOX38 is a transmissible F replicon made by recircularization of the large *F HindIII* fragment and lacks transposable sequences (6). pOX38-Km contains a *kan* gene insert at the pOX38 *HindIII* site (3). *F lac* and *F lac traW546* strains have been described elsewhere (14).

Host strains included XK1200 [*F<sup>-</sup> lac $\Delta$ U124  $\Delta$ (*nadA gal att $\lambda$  bio gyrA*)*], JC3051 [*F<sup>-</sup> lac $\Delta$ X74 his trp rpsL tsx ton ( $\lambda$ )*], and SE5000 [*F<sup>-</sup> araD139 lac $\Delta$ U169 rpsL relA thi recA56*] (20). N4830, which is *F<sup>-</sup>  $\lambda$  [cI857  $\Delta$ BAM( $\Delta$ 58.0-71.3%)  $\Delta$ H1( $\Delta$ cro R A J b2) bio *uvrB ilv]** (5), was used for pLa2311 derivatives. Electron microscopic observations of F pili were performed with the host VL584 [*F<sup>-</sup> ara thi  $\Delta$ (lac pro)  $\Delta$ (*uxu fimD*) rpsL*] (22). Media, growth, and mating conditions have been reported elsewhere (13).

**DNA cloning and sequencing.** In vitro DNA manipulations were performed according to standard protocols as described previously (13, 15). The *AatII-ScaI* adapter used to create a *ScaI* site in *trbI* was the self-complementary oligonucleotide 5'-AGTACTACGT-3'. Linker 1062, from New England Biolabs, Inc., Beverly, Mass., which contains a nonsense codon in all three reading frames, was used for the insert in pKI143. For nucleotide sequences, both DNA strands were determined by using the Sequenase system (United States Biochemical Corp.), M13mp8 and M13mp9 clones, and additional primers purchased from the Advanced

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TABLE 1. Plasmids

Plasmid	Fragment inserted <sup>a</sup>	Vector site used	<i>tra</i> gene(s) carried <sup>b</sup>	Comment or reference <sup>c</sup>
pKI139	1.7-kb <i>HincII</i>	pACYC177 <i>SmaI</i>	<i>trbI</i> , <i>traW546</i>	Like pKI144, but <i>traW</i> mutant DNA
pKI143	3-frame amber codon linker	pKI144 <i>SmaI</i>	<i>trbIam</i> , <i>traW</i>	Amber codons inserted in pKI144 <i>trbI</i>
pKI144	1.7-kb <i>HincII</i>	pACYC177 <i>SmaI</i>	<i>trbI</i> , <i>traW</i>	
pKI153	0.95-kb <i>SmaI-HincII</i>	pACYC177 <i>HincII</i>	<i>traW546</i>	Like pKI154, but <i>traW</i> mutant DNA (14)
pKI154	0.95-kb <i>SmaI-HincII</i>	pACYC177 <i>HincII</i>	<i>traW</i>	14
pKI157	1.7-kb <i>HincII</i>	pACYC177 <i>HincII</i>	<i>trbI</i> , <i>traW546</i>	Like pKI183, but <i>traW</i> mutant DNA (14)
pKI172	2.55-kb <i>AvaI</i>	pACYC177 <i>XmaI</i>	( <i>traC</i> , <i>trbI</i> )	14
pKI175	6.25-kb <i>SmaI</i>	pACYC177 <i>SmaI</i>	<i>traW</i> , <i>traU</i> , <i>trbC</i> , <i>traN</i> , <i>trbE</i> , <i>traF</i> , <i>trbA</i> , <i>artA</i> , <i>traQ</i>	29
pKI183	1.7-kb <i>HincII</i>	pACYC177 <i>HincII</i>	<i>trbI</i> , <i>traW</i>	14
pKI321	0.95-kb <i>SmaI-HincII</i>	pLa2311 <i>ScaI</i>	<i>traW</i>	
pKI457	1.7-kb <i>HincII</i>	pKI497	<i>trbI</i> , <i>traW</i>	
pKI463	1.25-kb <i>HincII</i> from pUC4K	pKI457 <i>SmaI</i>	<i>trbI463</i> , <i>traW</i>	<i>trbI::kan</i> insertion in <i>tra</i> orientation
pKI464	1.25-kb <i>HincII</i> from pUC4K	pKI457 <i>SmaI</i>	<i>trbI464</i> , <i>traW</i>	<i>trbI::kan</i> insertion in anti- <i>tra</i> orientation
pKI471	<i>AatII-ScaI</i> adapter	pKI457 <i>AatII</i>	<i>trbI</i> , <i>traW</i>	Create <i>ScaI</i> site at <i>trbI AatII</i>
pKI472	1.25-kb <i>HincII</i> from pUC4K	pKI471 <i>ScaI</i>	<i>trbI472</i> , <i>traW</i>	<i>trbI::kan</i> insertion in <i>tra</i> orientation
pKI473	1.25-kb <i>HincII</i> from pUC4K	pKI471 <i>ScaI</i>	<i>trbI473</i> , <i>traW</i>	<i>trbI::kan</i> insertion in anti- <i>tra</i> orientation
pSH1	13.85-kb <i>EcoRI</i>	RSF2124 <i>EcoRI</i>	<i>traC</i> , <i>trbI</i> , <i>traW</i> , <i>traU</i> , <i>trbC</i> , <i>traN</i> , <i>trbE</i> , <i>traF</i> , <i>trbA</i> , <i>artA</i> , <i>traQ</i> , <i>trbB</i> , ORF93, <i>trbF</i> , <i>traH</i> , ( <i>traG</i> )	Truncated <i>traG</i> product suffices in pilus expression (1)
pOX38	45.4-kb <i>HindIII</i>	None	Whole <i>tra</i> region	Tra <sup>+</sup> , circularized F fragment (6)
pOX38-Km	<i>kan HindIII</i>	pOX38 <i>HindIII</i>	Whole <i>tra</i> region	Tra <sup>+</sup> (3)
pOX38- <i>trbI463</i>	<i>trbI::kan</i>	pOX38	Whole <i>tra</i> region	In vivo recombination of pKI463 and pOX38
pOX38- <i>trbI464</i>	<i>trbI::kan</i>	pOX38	Whole <i>tra</i> region	In vivo recombination of pKI464 and pOX38
pOX38- <i>trbI472</i>	<i>trbI::kan</i>	pOX38	Whole <i>tra</i> region	In vivo recombination of pKI472 and pOX38
pOX38- <i>trbI473</i>	<i>trbI::kan</i>	pOX38	Whole <i>tra</i> region	In vivo recombination of pKI473 and pOX38

<sup>a</sup> Unless otherwise noted, the insert is a *tra* fragment, in the appropriate orientation for expression from the vector promoter.

<sup>b</sup> Parentheses indicate that part of the gene is missing.

<sup>c</sup> Unless otherwise cited, the plasmid was constructed for this study.

DNA Technology Laboratory (Biology Department, Texas A&M University). Both dGTP and dTTP reaction mixtures were examined. Computer analyses were performed by using the Genetics Computer Group sequence analysis software package, version 7.1 (March 1992); data bases searched were GenBank release 70.0 (December 1991), EMBL release 29.0 (December 1991), SwissProt release 20.0 (November 1991), and PROSITE release 8.0 (December 1991).

**Protein analysis.** The details of our protein analysis and fractionation procedures have been described elsewhere (13, 15). Plasmid products were labeled with [<sup>35</sup>S]methionine in SE5000 maxicells, separated on sodium dodecyl sulfate-polyacrylamide gradient gels, and identified by autoradiography. When noted, the maxicell samples were fractionated prior to analysis on the gels to obtain periplasmic proteins (15) or inner membranes (13).

**Nucleotide sequence accession number.** The nucleotide sequence shown in Fig. 2 has been assigned GenBank accession number M93106.

## RESULTS

**Nucleotide sequence of the *traW* region.** In previous work, we had shown that *traW546*-complementing activity was

expressed by plasmids carrying segments of F transfer region DNA derived from the *traC-traU* interval. The nucleotide sequence that we determined for this region is shown in Fig. 2. Nucleotides (nt) 1 to 364 in Fig. 2 are nt 2864 to 3227 of the *traC* sequence (accession number M30936) reported by Schandel et al. (23), and nt 993 is nt 1 of the *traU* sequence (accession number M34695) reported by Moore et al. (18). The first open reading frame in the sequence which extends for 128 codons has been named *trbI*. Hydrophobicity analysis of the *M<sub>r</sub>*-14,132 product *trbI* encodes suggested that TrbI includes a membrane-spanning region extending from amino acids 17 to 56 (12). Since the 0.95-kb *SmaI-HincII* fragment carried by pKI154 expresses *traW*-complementing activity (14) (Fig. 1), we could identify the second open reading frame in Fig. 2 as *traW*. In confirmation, we found that this 210-codon open reading frame is interrupted in *traW546* mutant DNA as a result of a change that alters amino acid codon 141, CAG (Gln), to the amber stop codon TAG. Analysis of the *M<sub>r</sub>*-23,630 product encoded by *traW* suggested there was a signal peptide at the amino terminus of the gene product. By the -3,-1 rule (25), processing is predicted to occur either between amino acids 17 and 18 or between amino acids 19 and 20. Such processing would

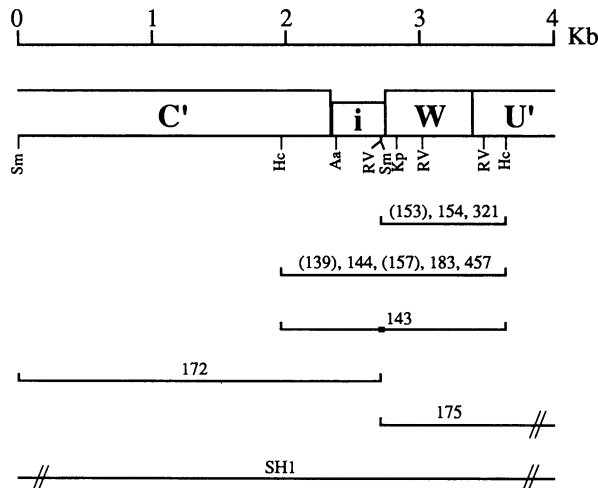


FIG. 1. Map of an F transfer region segment containing *trbI* and *traW*. Boxes containing letters indicate the positions and sizes of *trbI* (i), *traW* (W), and portions of the flanking genes *traC* (C') and *traU* (U'). Numbers on lines below the map correspond to pKI plasmid clones of the segments delineated; // indicates that the cloned segment extends beyond the region shown. Numbers in parentheses indicate that the clone carries *traW546* mutant DNA. pKI143 contains an amber codon linker insertion (small box). Restriction sites: Aa, *AatII*; Hc, *HincII*; Kp, *KpnI*; RV, *EcoRV*; Sm, *SmaI*.

result in a hydrophilic mature polypeptide of either 21,760 or 21,618 Da.

Figure 2 also shows that the four contiguous genes, *traC*, *trbI*, *traW*, and *traU*, each overlap slightly; an ATGA sequence encodes both the translational stop codon for the upstream gene and the translational initiation codon of the downstream gene at the *traC-trbI*, *trbI-traW*, and *traW-traU* junctions.

**In vivo processing of the *traW* product.** Since the DNA sequence of *traW* suggested that its product might be processed, we examined the effect of ethanol on the proteins expressed from *traW* clones. Figure 3 shows the results of a pulse-chase experiment in which products of the TraW<sup>+</sup> plasmids pKI144 and pKI154 were compared with those of the similarly constructed *traW546* amber mutant plasmids pKI139 and pKI153. Maxicells carrying these plasmids were labeled with [<sup>35</sup>S]methionine for 15 min in the presence and absence of 9% ethanol. A portion of each ethanol sample was then washed and incubated for 30 min more with an excess of unlabeled methionine. In the absence of ethanol (Fig. 3, lanes A), the TraW<sup>+</sup> plasmids expressed the M<sub>a</sub>-23,000 product previously identified as TraW. When these maxicells were incubated with ethanol (lanes E), this product disappeared and an M<sub>a</sub>-25,000 product accumulated. However, after the chase period of incubation without ethanol, the M<sub>a</sub>-23,000 mature protein could be detected in these samples (lanes C). The TraW<sup>-</sup> amber mutant derivatives did not express either of these proteins. Similar results were obtained in experiments with plasmids pKI183 and pKI157, although in this case, distinction of the TraW precursor was more difficult because of a 24.4-kDa fusion protein containing sequences from the β-lactamase N terminus and the C terminus of TraC that also accumulated in the ethanol samples (data not shown). We concluded that *traW* does express a precursor protein that undergoes signal sequence processing.

**Subcellular location of TraW.** The hydrophilic properties of the product encoded by *traW* suggested that after processing, the mature protein would localize in the periplasmic space. Indeed, in examining the location of the *traF* product, Wu et al. (28) examined periplasmic fractions from cells containing the large plasmid, pKI175, and noted the presence of the 23-kDa protein we have identified as TraW. To confirm the subcellular location of TraW, we labeled maxicells carrying pKI144 and examined periplasmic fractions prepared by two other different procedures. Proteins released by osmotic shock (lane 0) or obtained by chloroform extraction (lane Cf) are compared with an untreated maxicell sample (lane M) in Fig. 4. Both the *traW* product and β-lactamase were present in both types of periplasmic protein fractions, made from a culture of the N4830/pKI321 strain that had been induced and grown at 42°C for 4 h, contained a large quantity of TraW which could be detected on a Coomassie blue-stained gel (data not shown). Thus, we concluded that TraW is a periplasmic protein.

**Electron microscopy of *F lac* and *F lac traW546* strains.** Since *F lac traW546* hosts are resistant to F-pilus-specific phages, Miki et al. (17) tentatively classified *traW* as an F-pilus assembly gene. However, *traU* mutants, which have a similar phenotype, were recently found to express pili (18). To more conclusively test whether the phage resistance caused by *traW546* reflects an absence of pili, we examined the properties of an *F lac traW546* strain by electron microscopy, using the host VL584, which does not express other surface appendages. In a VL584/*F lac* wild-type control culture, F pili could be observed on 52% of the cells, and there was an overall average of 0.64 pili per cell. No F pili were observed on the 100 VL584/*F lac traW546* cells examined. However, introduction of the TraW<sup>+</sup> plasmid pKI154 into the VL584/*F lac traW546* strain restored pilus production as well as phage sensitivity; 29% of the cells observed were piliated, and there was an overall average of 0.39 pili per cell. Since F pilin has been detected in membranes of an *F lac traW546* strain (19), our findings confirm that F-pilus assembly is dependent on *traW*.

**Identification of the *trbI* product.** The product of *trbI* was identified by comparison of the [<sup>35</sup>S]methionine-labeled products synthesized in maxicells containing plasmid clones of the complete *trbI* reading frame (pKI144 and pKI139) or an incomplete *trbI* reading frame (pKI172 and pKI154). As shown in Fig. 5A, pKI144 and pKI139 expressed a 14-kDa polypeptide that was not synthesized by pKI172, pKI139, or the parental vector pACYC177. To confirm the origin of this polypeptide, plasmid pKI143 was constructed by inserting a stop codon linker at the unique *SmaI* site in the *trbI* sequence of pKI144. Plasmid pKI143 was unable to express the 14-kDa polypeptide (Fig. 5B). The stop codon linker insertion also exhibited polarity in pKI143, reducing *traW* expression to less than the detectable level. These results firmly demonstrated that the *trbI* sequence is expressed as a 14-kDa protein.

**Subcellular location of the *trbI* product.** After labeling of pKI144 products with [<sup>35</sup>S]methionine, maxicells were first fractionated as described by Wu et al. (28) to determine the

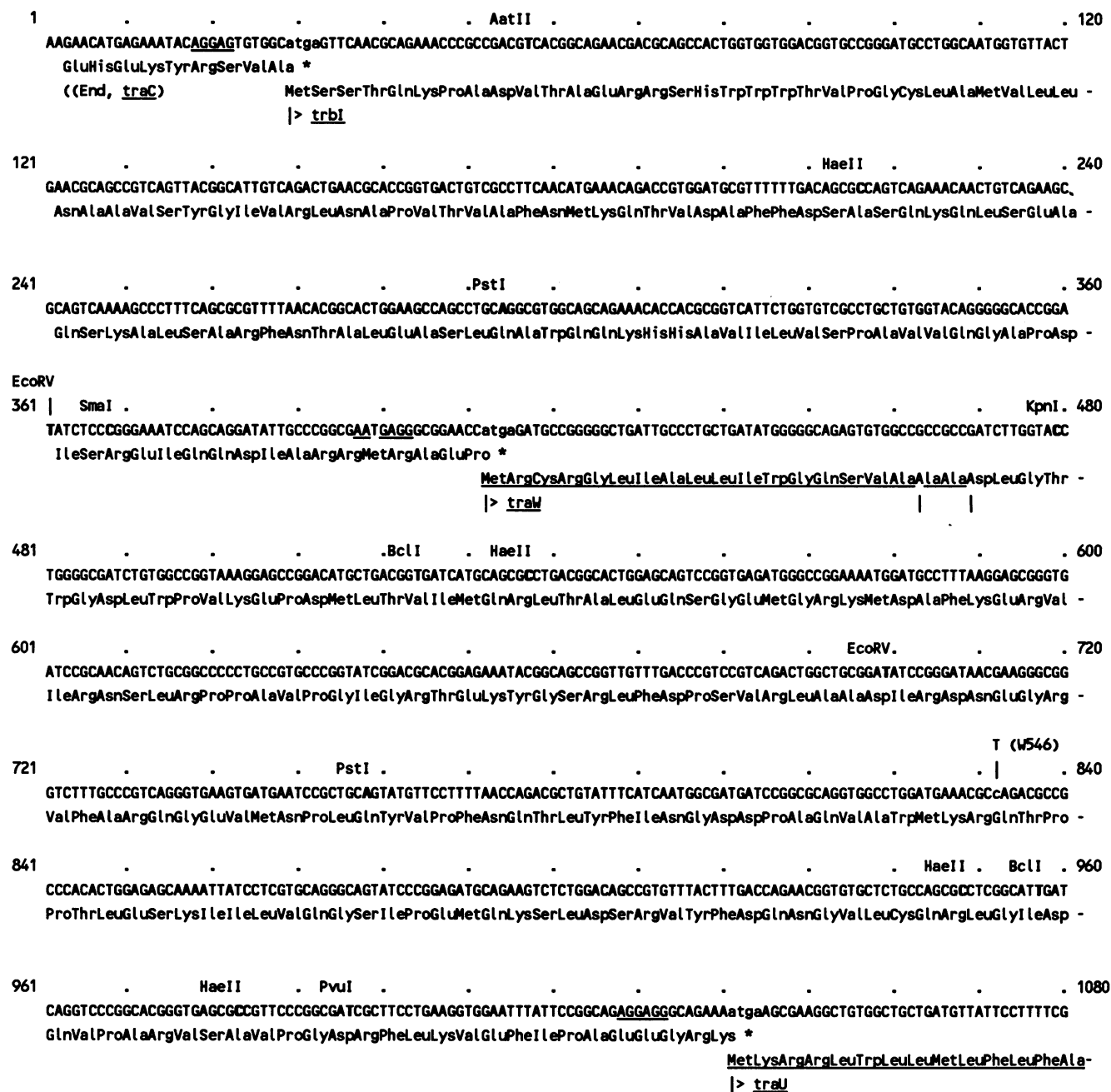


FIG. 2. Nucleotide sequences of the *trbI* and *traW* genes. The translation products for *trbI* and *traW* and the flanking *trac* and *traU* sequences are also shown. Translation start and stop codons are shown in lowercase. Potential ribosome binding sites and precursor membrane signal sequences are underlined; vertical lines indicate two alternative possibilities for the amino terminus of mature TraW. The positions of the C→T transition found in *traW546* amber mutant DNA and of selected restriction sites are also indicated. The sequence shown begins with nt 2864 to 3227 of the sequence reported by Schandel et al. (23) and ends with nt 1 to 88 of that reported by Moore et al. (18).

location of TrbI. Figure 6A shows that unlike  $\beta$ -lactamase, which was enriched in the periplasmic fraction (lane P), the *trbI* product was enriched in the membrane fraction (lane M). The *trbI* protein remained in the membrane pellet fraction after extraction with  $\text{Na}_2\text{CO}_3$  to remove peripheral membrane proteins but could be readily solubilized with Triton X-100 and recovered by trichloroacetic acid precipitation of the detergent supernatant fraction (data not shown). Sucrose density gradient fractionation of the labeled maxi-

cells confirmed the compartmentalization of TrbI (Fig. 6B). The labeled *trbI* product was enriched in fractions 5 and 6, which are at the interface between 15 and 53% sucrose in the step gradient that we used (10). Coomassie blue staining of the gel showed that outer membrane proteins were primarily in fractions 1 and 2. Thus, all of our data, including hydrophathy analysis of the *trbI* product, indicate that TrbI is an integral inner membrane protein.

Construction and analysis of pOX38-*trbI* mutants. We

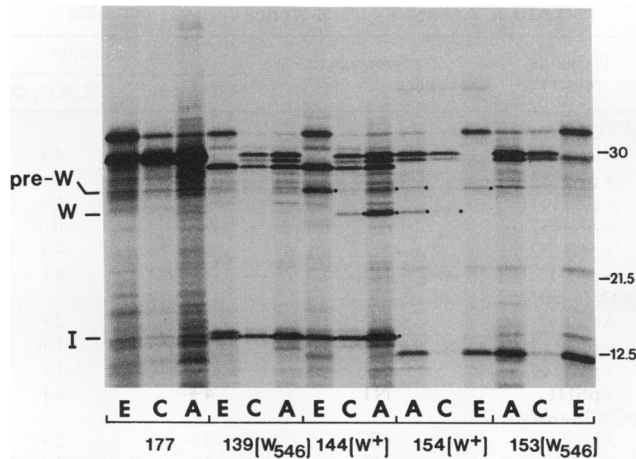


FIG. 3. In vivo processing of the *traW* product. An autoradiogram showing the [<sup>35</sup>S]methionine-labeled products of maxicells carrying *TraW*<sup>+</sup> and *traW546* mutant plasmids (indicated as *W*<sup>+</sup> or *W*<sub>546</sub>) is shown. Numbers below the lanes indicate the plasmid present and correspond to that of the vector, pACYC177, or of plasmids pKI139, pKI144, pKI153, and pKI154. Samples were labeled in the absence of ethanol (lanes A), in the presence of ethanol (lanes E), or with ethanol followed by an ethanol-free chase period (lanes C). The positions of the *TraW* precursor protein (pre-W), mature *TraW* (W), and the *trbI* product (I) are indicated on the left; numbers at the right show the positions and sizes (in kilodaltons) of molecular weight markers on the stained gel.

investigated the function of *trbI* by constructing *trbI* kanamycin insertion mutations in pOX38, a transmissible F-deletion derivative that retains all *tra* region sequences. The approach used has been effective in analysis of other *tra* operon gene functions (11, 13, 15, 18). In this case, we first constructed pKI457, a clone carrying the 1.7-kb *HincII* fragment that includes *trbI* and *traW*. Since the parental vector (pKI497) lacks *SmaI* and *AatII* sites, these occur uniquely in the pKI457 *trbI* sequence and could be used to insert a pUC4K *HincII* fragment encoding a *kan* gene into *trbI*. The four insertion mutations that we obtained are diagrammed in Fig. 7. Each was then crossed onto pOX38, using the triparental mating procedure and selections described previously (11, 15). Restriction analysis of four representative recombinant plasmids, pOX38-*trbI463*, pOX38-*trbI464*, pOX38-*trbI472*, and pOX38-*trbI473*, confirmed that they carry the four *trbI::kan* insertions made in

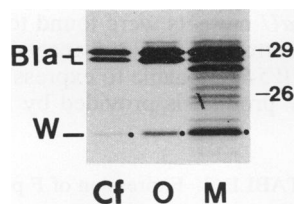


FIG. 4. Proteins in periplasmic fractions of [<sup>35</sup>S]methionine-labeled maxicells carrying pKI144. Periplasmic protein samples were obtained by chloroform treatment (lane Cf) or osmotic shock treatment (lane O). An unfractionated maxicell sample (lane M) is also shown. Bla and W indicate the positions of the mature β-lactamase and *TraW* products; positions and sizes (in kilodaltons) of molecular weight marker proteins are indicated on the right.

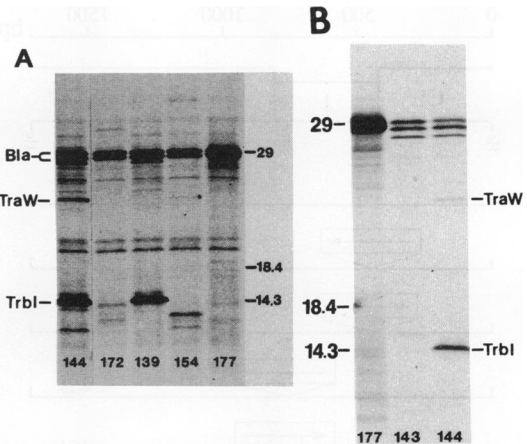


FIG. 5. Identification of the *trbI* protein. Autoradiograms show the [<sup>35</sup>S]methionine-labeled products of maxicells carrying various plasmids. Lane numbers in panel A correspond to plasmids pKI144, pKI172, pKI139, pKI154, and pACYC177; those in panel B correspond to plasmids pKI144, pKI143, and pACYC177. The positions of the β-lactamase (Bla), *TraW*, and *TrbI* proteins and the sizes (in kilodaltons) and positions of molecular weight marker proteins are indicated.

plasmids pKI463, pKI464, pKI472, and pKI473, respectively.

We tested the transfer efficiency and pilus-specific phage sensitivity of hosts carrying each of the pOX38 *trbI* insertion mutants and examined the appearance of some of these strains by transmission electron microscopy. These results, together with complementation results obtained after introduction of *trbI* clones into these strains, are summarized in Tables 2 and 3. All of the *trbI* mutant plasmids could transfer relatively efficiently, at levels 40 to 100% of that obtained with the wild-type pOX38-Km control. In phage spot tests, the *SmaI* site *kan* insertions pOX38-*trbI463* and pOX38-*trbI464* retained normal sensitivity (4+) to DNA phages but exhibited reduced sensitivity (2+) to the RNA phages. The *AatII* site insertion derivatives, pOX38-*trbI472* and pOX38-*trbI473*, were, however, only partially sensitive (2+) to the DNA phages and were very insensitive (1+) to the RNA phages. Strains carrying these mutant plasmids expressed

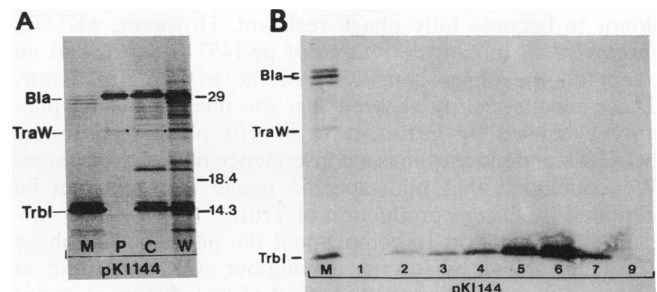


FIG. 6. Subcellular location of *TrbI*. Proteins were labeled in maxicells carrying pKI144. Products and molecular weight markers are indicated as in Fig. 5. (A) Samples contained a membrane fraction (lane M), a periplasmic protein fraction (lane P), a cytoplasmic protein fraction (lane C), and unfractionated maxicells (lane W). (B) Lane M contained unfractionated maxicells; lanes 1 to 9 contained fractions of decreasing density obtained by dripping 1-ml samples from the bottom of a sucrose gradient.

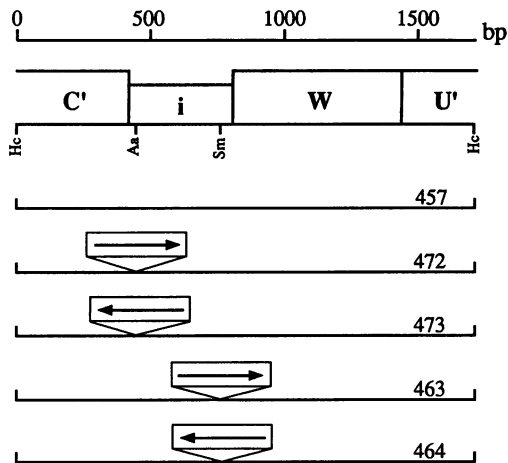


FIG. 7. Insertion mutations in *trbI*. A map of the *tra* region *HincII* fragment carried by pKI457 is shown. Symbols are as given for Fig. 1. Lines below the map show *tra* DNA segments in correspondingly numbered pKI plasmids; boxes containing arrows indicate the direction of *kan* expression and position of *kan* insertions at the *AatII* (Aa) and *SmaI* (Sm) sites in *trbI*. The *kan* gene cassette used was from pUC4K and is not drawn to scale.

reasonably normal numbers of F pili that could adsorb RNA phages (Table 3). However, the pili were unusually long, extending three to five times farther from the cell surface than did wild-type pili (Fig. 8).

We next tested whether normal phage sensitivity could be restored to the *trbI* mutant hosts through complementation from clones that express *trbI*. Surprisingly, we found that introduction of a multicopy plasmid carrying *trbI* alone (pKI139) or carrying *trbI* and *traW* (pKI144) caused the *trbI* mutant strains to become even more phage resistant. Furthermore, these plasmids also reduced the pilus-specific phage sensitivity of the wild-type control strain carrying pOX38-Km, although they did not affect the frequency of pOX38-Km transfer. Since introduction of the parental vector, pACYC177, had no effect on the phage sensitivity of the pOX38-Km donor, we explored this effect further and tested the effect of introducing pKI457. This plasmid, which expresses *trbI* more efficiently than do pKI139 and pKI144 because of its higher copy number, caused the wild-type donor to become fully phage resistant. However, pKI472, the *trbI::kan* mutant derivative of pKI457 (Fig. 7), had no effect on the phage sensitivity of the pOX38-Km donor. These results clearly showed that the phage-resistant phenotype caused by introduction of *trbI* plasmids into the pOX38-Km donor strain is a consequence of *trbI* expression. We concluded that pilus-specific phage infection can be inhibited by excess production of TrbI.

In a further effort to complement the pilus-specific phage resistance shown by strains carrying our pOX38-*trbI* mutant derivatives, we also tested the effect of introducing plasmids carrying additional *tra* genes. Phage sensitivity increased when pKI175 was introduced into pOX38-*trbI472* and pOX38-*trbI473* strains. This plasmid does not express *trbI* but does express all of the genes in the 6.45-kb *SmaI* fragment distal to *trbI* (*traW* to *traQ*). Full sensitivity was restored when pSH1, which expresses genes *traC* to *traG* (including *trbI*), was introduced into any of the pOX38-*trbI* mutant strains.

TABLE 2. Phenotypes of strains carrying plasmids

Plasmid(s) present <sup>a</sup>	Transconjugants/100 donor cells <sup>b</sup>	Phage sensitivity <sup>c</sup>	
		f1, M13, fd	f2, R17, Q $\beta$
<b>pOX38-Km<sup>d</sup></b>			
Alone	60	4+	4+
+pKI139	67	2+	1+
+pKI144	53	2+	1+
+pKI457	42	R	R
+pKI472	NT <sup>e</sup>	4+	4+
+pSH1	NT	4+	4+
<b>pOX38-<i>trbI463</i></b>			
Alone	53	4+	2+
+pKI139	NT	2+	1+
+pKI144	NT	2+	1+
+pSH1	NT	4+	4+
<b>pOX38-<i>trbI464</i></b>			
Alone	60	4+	2+
+pKI144	NT	2+	1+
+pSH1	NT	4+	4+
<b>pOX38-<i>trbI472</i></b>			
Alone	30	2+	1+
+pKI139	NT	1+	R
+pKI144	40	1+	R
+pKI175	NT	3+	3+
+pSH1	NT	4+	4+
<b>pOX38-<i>trbI473</i></b>			
Alone	20	2+	1+
+pKI139	NT	1+	R
+pKI144	33	1+	R
+pKI175	NT	3+	3+
+pSH1	NT	4+	4+

<sup>a</sup> XK1200 derivatives.

<sup>b</sup> Km<sup>r</sup> Str<sup>r</sup> after mating with JC3051.

<sup>c</sup> Phage spot tests: 4+, fully sensitive; 3+, sensitive with some growth in spot; 2+, slightly sensitive, turbid spot; 1+, lysis detected at edge of spot; R, no lysis detectable.

<sup>d</sup> Tra<sup>+</sup> control.

<sup>e</sup> NT, not tested.

## DISCUSSION

Previous studies have shown that a number of F *tra* region products undergo signal sequence processing and localize in the periplasmic space. These include proteins encoded by *traU* (18), *trbC* (15), *traF* (28), *trbB* (27), and, most probably, *traH* (7). Both the predicted amino acid sequence and the properties of the *traW* products that we have identified demonstrate that TraW is also a member of this group of proteins. Like most of these products (TrbB is the exception), TraW is required for conjugative transfer and F-pilus-specific phage sensitivity (17); since defects in *traW* or other genes in this group do not block F-pilin subunit synthesis (11, 19), a requirement in F-pilus assembly has been implied. Nevertheless, *traU* mutants were found to express some F pili (18). Our electron microscope analysis shows that the mutant F *lac traW546* is unable to express F-pilus filaments unless the TraW product is provided by a complementing

TABLE 3. Expression of F pili

Plasmid present <sup>a</sup>	% Cells with F pili	Avg no. of F pili per cell
pOX38-Km	64	1.0
pOX38- <i>trbI472</i>	59	0.77
pOX38- <i>trbI473</i>	58	0.66

<sup>a</sup> In strain VL584.

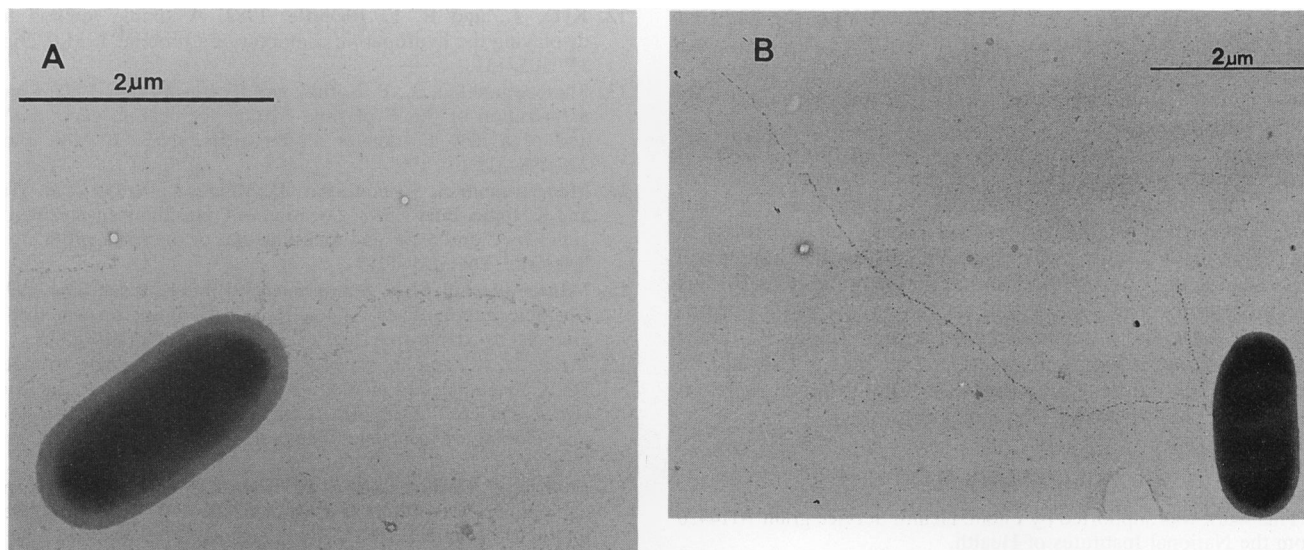


FIG. 8. F pili expressed by a *trbI* mutant. R17 bacteriophages are adsorbed to the F-pilus filaments. (A) Wild-type pilus expression from a VL584/pOX38 cell; (B) representative cell and pili seen during examination of strains VL584/pOX38-*trbI472* and VL584/pOX38-*trbI473*.

plasmid and demonstrates conclusively that TraW is essential for F-pilus assembly. TraW interactions with other F-pilus assembly proteins remain to be explored.

Our studies also showed that there is a previously uncharacterized gene, *trbI*, in the interval between *traC* and *traW*. Our *trbI* sequence encodes a 14,132-Da product with membrane-spanning potential. These features are entirely consistent with the properties of the 14-kDa intrinsic inner membrane protein product that we have characterized. Recent analysis of a *trbI::phoA* fusion product also confirms the membrane-spanning potential of TrbI sequences. Schandel et al. (24) report that a TrbI amino-terminal sequence permits alkaline phosphatase translocation to the periplasm, although the TrbI::alkaline phosphatase polypeptide remains associated with membrane fractions.

Our finding that hosts containing the transmissible F derivative pOX38-Km became more resistant to F-pilus-specific phages when small multicopy *trbI* plasmids were introduced was interesting. This effect was clearly related only to *trbI* expression and appeared to correlate with the amount of TrbI expressed; the highest-copy-number plasmid caused the most profound resistance. Thus, we conclude that an excess of TrbI can interfere with the infective process of these phages. Both single-stranded DNA phage and RNA phage infections were affected by the *trbI* clones, suggesting that TrbI excess can block an interaction or event that is required by both phage types. Although a preliminary electron microscopic examination of the F pili expressed by cells carrying both pKI457 and pOX38 revealed no abnormality (data not shown), a more detailed examination of these strains should prove to be interesting; a TrbI effect on the outgrowth and retraction kinetics of pili or on the release or entry of phage nucleic acids into the cell seems likely. However, we have not ruled out the possibility that TrbI interferes at a later stage, such as the phage maturation process.

The pOX38-*trbI::kan* mutants that we constructed were able to transfer quite efficiently, indicating that *trbI* is not essential for conjugative DNA transfer under standard mating conditions. However, the *trbI* mutations also caused a

reduction in host sensitivity to both types of pilus-specific phages. We found that the insertion mutations *trbI472* and *trbI473* closest to the amino-terminal region of *trbI* (at the *AatII* site) had the stronger effect on phage sensitivity; these mutations also caused expression of unusually long pilus filaments, suggestive of a pilus retraction defect. We could not show that this phenotype is related solely to the absence of *trbI*, since expression from our clones that carried only *trbI* exacerbated the phage resistance of these strains as well as reducing the phage sensitivity of the wild-type control. While pSH1 restored the wild-type phenotype, this plasmid expressed not only *trbI* but also *traC* and all of the *trbI* distal genes required for pilus assembly. Furthermore, pKI175, which includes only a set of genes distal to *trbI*, did improve the phage sensitivity of the mutants.

One explanation of these findings is that the *trbI::kan* insertions exert a polar effect, reducing expression of downstream genes. The more distal insertion mutations in *trbI* (at the *SmaI* site) did have a weaker effect on the phage sensitivity phenotype. However, this effect could also reflect expression of a truncated TrbI product with some residual activity. Since the mutant strains did express F pili, they cannot entirely lack such gene products as TraW, TrbC, or TraQ since these products are required for expression of these filaments. Furthermore, *kan* insertions in *traU*, *trbC*, *trbA*, *artA*, or *traQ* do not result in a polar phenotype, nor do amber mutations in *traW*, *traU*, *traN*, or *traF* (11, 13–15, 18, 28); all such mutations are also fully complemented by pKI175. Our TrbI<sup>+</sup> TraW<sup>+</sup> clones also increased, rather than alleviated, the phage resistance of the *trbI::kan* mutants. Nevertheless, it is possible that the gene products distal to *trbI* are expressed at lower levels in our mutant strains and that, to some extent, the *trbI* mutant phenotype depends on this effect.

A second explanation, implied by the effect of *trbI* expression on the wild-type plasmid phenotype, is that the ratio of TrbI to one or more of the other *tra* products is important and can affect the course of phage infection. We suggest that TrbI may affect the extension and/or retraction of F pili. Although further experiments will be required to determine the stage at

which phage infection is inhibited in our strains, the fact that our *trbI* mutants resemble some of the F *lac* mutants described by Burke et al. (2) is very intriguing. They described three isolates which also made long pili and retained a high level of transfer proficiency as well as some sensitivity to pilus-specific phages. Each displayed one or more defects in their capacity to retract pili in response to cyanide or to heat or in their ability to regenerate pili after blending. Our *trbI::kan* mutations are the first genetically characterized F *tra* region lesions known to enhance pilus length. Thus, it will be of significant interest to examine the physiology of F-pilus assembly and retraction as well as mating aggregate properties with these and other *trbI* mutant strains. Similarly, donor strains carrying a high-copy-number *trbI* plasmid should be useful in such studies since these are totally phage resistant but retain a high transfer frequency.

#### ACKNOWLEDGMENTS

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

We appreciate the assistance of R. W. Davis with electron microscopy.

#### REFERENCES

- Achtman, M., R. A. Skurray, R. Thompson, R. Helmuth, S. Hall, L. Beutin, and A. J. Clark. 1978. Assignment of *tra* cistrons to *EcoRI* fragments of sex factor DNA. *J. Bacteriol.* **133**:1383-1392.
- Burke, J. M., C. P. Novotny, and P. Fives-Taylor. 1979. Defective F pili and other characteristics of *Flac* and *Hfr* *Escherichia coli* mutants resistant to bacteriophage R17. *J. Bacteriol.* **140**:525-531.
- 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.
- 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.
- Gottesman, M. E., S. Adhya, and A. Das. 1980. Transcription antitermination by bacteriophage lambda N gene product. *J. Mol. Biol.* **140**:57-75.
- 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  $\gamma\delta$ . *Cold Spring Harbor Symp. Quant. Biol.* **45**:135-140.
- 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.
- Ippen-Ihler, K., and S. Maneewannakul. 1991. Conjugation among enteric bacteria: mating systems dependent on expression of pili, p. 35-69. *In* M. Dworkin (ed.), *Microbial cell-cell interactions*. American Society for Microbiology, Washington, D.C.
- 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.
- Ito, K., T. Sato, and T. Yura. 1977. Synthesis and assembly of the membrane proteins in *E. coli*. *Cell* **11**:551-559.
- Kathir, P., and K. Ippen-Ihler. 1991. Construction and characterization of derivatives carrying insertion mutations in F plasmid transfer region genes *trbA*, *artA*, *traQ*, and *trbB*. *Plasmid* **26**:40-54.
- Kyte, J., and R. F. Doolittle. 1982. A simple method for displaying the hydropathic character of a protein. *J. Mol. Biol.* **157**:105-132.
- Maneewannakul, S., P. Kathir, and K. Ippen-Ihler. 1992. Characterization of the F plasmid mating-aggregation gene, *traN*, and of a new F transfer region locus, *trbE*. *J. Mol. Biol.* **225**:299-311.
- 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.
- Maneewannakul, S., K. Maneewannakul, and K. Ippen-Ihler. 1991. Characterization of *trbC*, a new F plasmid *tra* operon gene that is essential to conjugative transfer. *J. Bacteriol.* **173**:3872-3878.
- 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.
- Miki, T., T. Horiuchi, and N. S. Willetts. 1978. Identification and characterization of four new *tra* cistrons on the *E. coli* K12 sex factor F. *Plasmid* **1**:316-323.
- 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.
- 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.
- 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.
- Remaut, E., P. Stanssens, and W. Fiers. 1981. Plasmid vectors for high efficiency expression controlled by the P<sub>L</sub> promoter of coliphage lambda. *Gene* **15**:81-93.
- Schandel, K. A., S. Maneewannakul, K. Ippen-Ihler, and R. E. Webster. 1987. A *traC* mutant that retains sensitivity to fl bacteriophage but lacks F pili. *J. Bacteriol.* **169**:3151-3159.
- 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.
- Schandel, K. A., M. M. Muller, and R. E. Webster. Localization of TraC: a protein involved in the assembly of the F conjugative pilus. *J. Bacteriol.* **174**:3800-3806.
- von Heijne, G. 1987. *Sequence analysis in molecular biology: treasure trove or trivial pursuit*. Academic Press, Inc., London.
- 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.
- 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.
- 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.
- 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.