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The *Escherichia coli* K-12 F Plasmid Gene *traX* Is Required for Acetylation of F Pilin

DEANNA MOORE,[1] CHARLEEN M. HAMILTON,[1]† KESMANEE MANEEWANNAKUL,‡[1] YAEL MINTZ,[1] LAURA S. FROST,[2] AND KARIN IPPEN-IHLER[1]*

Department of Medical Microbiology and Immunology, Texas A&M University, College Station, Texas 77843,[1] and Department of Microbiology, University of Alberta, Edmonton, Alberta, Canada T6G 2E9[2]

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The *Escherichia coli* F plasmid gene required for amino-terminal acetylation of F-pilin subunits was identified. Using Western blots (immunoblots), we assayed the reaction of monoclonal antibodies with F-pilin polypeptides in inner membrane preparations from various F mutant strains. It was known that JEL92 recognizes an internal pilin epitope and JEL93 recognizes the acetylated amino-terminal sequence (L. S. Frost, J. S. Lee, D. G. Scraba, and W. Paranchych, J. Bacteriol. 168:192–198, 1986). As expected, neither antibody reacted with inner membranes from F^- cells or F*lac* derivatives that do not synthesize pilin. Mutations that affected the individual activities of F *tra* genes *traA*, *-B*, *-C*, *-D*, *-E*, *-F*, *-G*, *-H*, *-I*, *-J*, *-K*, *-L*, *-M*, *-N*, *-P*, *-R*, *-U*, *-V* and *-W* or *trb* genes *trbA*, *-B*, *-C*, *-D*, *-E*, *-G*, *-H*, and *-I* did not prevent JEL92 or JEL93 recognition of membrane pilin. However, Hfr deletion mutants that lacked the most-distal transfer region genes did not express pilin that reacted with JEL93. Nevertheless, all strains that retained *traA* and *traQ* did express JEL92-reactive pilin polypeptides. Analysis of strains expressing cloned *tra* segments showed that *traA* and *traQ* suffice for synthesis of JEL92-reactive pilin, but synthesis of JEL93-reactive pilin is additionally dependent on *traX*. We concluded that the *traX* product is required for acetylation of F pilin. Interestingly, our data also showed that $TraA^+$ $TraQ^+$ cells synthesize two forms of pilin which migrate at approximately 7 and 8 kDa. In $TraX^+$ cells, both become acetylated and react with JEL93. Preparations of wild-type F-pilus filaments contain both types of subunits.

Strains carrying the *Escherichia coli* K-12 conjugative plasmid, F, elaborate long filamentous appendages known as F pili, which extend from the cell surface and initiate mating contacts with recipient cells. Although a large number of F transfer region genes are required for F-pilus assembly, studies of purified F pili have identified only a single type of subunit, F pilin (for a review see references 17, 19, 40, and 47). This 7.2-kDa protein derives from the F *traA* gene which encodes a 121-amino-acid (13-kDa) precursor polypeptide; mature F pilin includes only the 70 C-terminal amino acids of that precursor and has an acetylated amino terminus (10). The acetylated amino terminus is the major antigen of F pilin (5, 7, 10).

A second gene essential to F-pilin synthesis was discovered by Moore et al. (39). Subsequent studies (18, 23) showed that if *traA* was expressed in hosts lacking *traQ*, the 13-kDa pilin precursor protein was the major *traA* product; a minor 7-kDa product was seen only when *traA* was expressed at high levels. In $TraQ^+$ cells, however, the 13-kDa *traA* product was observed only under conditions of overproduction. Instead, the major *traA* product was a 7-kDa polypeptide [named *traA* product 7(Q) or Ap7(Q)] that was expressed very efficiently (23). Laine et al. also detected small amounts of an 8-kDa *traA* product (Ap8) (23). Cells carrying an F*lac traQ* mutant plasmid have since been shown to be severely transfer deficient and unable to express F pili or detectable quantities of F-pilin subunits (22). Thus, the *traQ* product, a 94-amino-acid inner membrane protein (48, 49), appears to be required for efficient utilization and processing of the pilin precursor. It was suggested that Ap8 might be an intermediate in such processing (23).

Laine et al. (23) also showed that a third F gene product was needed for synthesis of mature F-pilin subunits. The 7-kDa pilin polypeptide [7(Q)] expressed from $TraA^+$ $TraQ^+$ lambda transducing phages in an F^- host migrated slightly faster than wild-type pilin and reacted very poorly with anti-F-pilus serum. When an F*lac traA* host was used, the pilin expressed from the phages comigrated with wild-type pilin and was readily precipitable with the serum. In an F^- host, plasmids expressing cloned *traA* and *traQ* sequences also expressed the faster-migrating polypeptide, 7(Q) (49). Thus, it appeared that the product of a third F gene modified the pilin polypeptide. Laine et al. (23) suggested that this additional F gene product might be required for acetylation of the pilin amino terminus and that the 7(Q) polypeptide lacked this immunodominant epitope (5, 7, 10).

Subsequently, Frost et al. (9) characterized two monoclonal antibodies that react with F pilin. One, JEL92, recognized an internal epitope near the amino terminus of F pilin centered around the Met-9 residue of the subunit. The other, JEL93, was shown to be specific for the blocked F-pilin amino terminus. Tests of its reaction with synthetic peptides corresponding to the first eight amino acid residues of F pilin showed that it was 100-fold-less reactive with the unacetylated form of the peptide [NH_2-F(1-8)] than with the acetylated form [Ac-F(1-8)]. In this study we have used JEL92 and JEL93 to identify the F locus responsible for converting the F-pilin polypeptide to its mature, acetylated form.

* Corresponding author.
† Present address: Department of Cell Biology and Neuroscience, University of Texas Southwestern Medical Center at Dallas, Dallas, TX 75235.
‡ Present address: Department of Biological Chemistry and Molecular Pharmacology, Harvard Medical School, Boston, MA 02115.

TABLE 1. Plasmids

Plasmid	Description
pKI150	*Hin*cII-*Sca*I pKI158 fragment (containing 0.44-kb *Sau*3AI-*Sma*I *traQ* fragment) in pACYC177 *Sma*I; Ap^r (35).
pKI158	0.44-kb *Sau*3AI-*Sma*I *traQ* fragment in pACYC177 *Hin*cII site; Km^r (49).
pKI301	1.45-kb *Pst*I *traALE* fragment in pLa2311 *Pst*I site; Km^r (23).
pKI356	*Hin*cII-*Sca*I pKI158 fragment (containing 0.44-kb *Sau*3AI-*Sma*I *traQ* fragment) in *Sca*I site, and 2.54-kb *Hin*cII *traJYAL* fragment in *Xmn*I site of pLa2311; Km^r (34).
pKI358	*Hin*cII-*Sca*I pKI158 fragment (containing 0.44-kb *Sau*3AI-*Sma*I *traQ* fragment) in *Sca*I site, and 1.45-kb *Pst*I *traALE* fragment in *Pst*I site of pLa2311; Km^r (34).
pKI481	*Acc*I-*Hin*dIII *traX* fragment expressed from T7 late promoter of pKI487 (24, 25).
pKM69	*Dsa*I-*Hin*dIII *traX* fragment expressed from a *lac* promoter; p15A replicon; Ap^r (24).
pOX38	45.5-kb F *Hin*dIII fragment circularized; Tra^+ (14).
pOX38-Km	Tn*5* *kan* *Hin*dIII fragment in pOX38 *Hin*dIII site; Tra^+ Km^r (2).
pPM55	$TraS^-$ T^- D^- I^- deletion derivative of pRS31; deletion endpoint at F coordinate 94.5; Tc^r (30, 44).
pRS31	*Eco*RI fragments f17-f19-f2 (*traSTDIX*) in pSC101 *Eco*RI site; Tc^r (42).
pTG801	20-kb F DNA extending from *traY* *Pst*I site to *traG* *Eco*RI site in pUC19 (13).

MATERIALS AND METHODS

Bacterial strains and plasmids. Plasmids carrying cloned *tra* segments are described in Table 1. Vectors used included pACYC177 (3) and pLa2311 (41). F*lac* and pOX38 derivatives carrying *tra* mutants are described in Table 2. Host strains for these plasmids were JC3272 (F^- *lacΔX74 gal his trp lys rpsL tsx*), ED2149 [F^- *lacΔU124* Δ(*nadA aroG gal attλ bio*)], and XK1200 [F^- *lacΔU124* Δ(*nadA aroG gal attλ bio*) *gyrA*] (38). The origins and properties of the Hfr deletion strains were described previously (16, 33). Strain N4830 [F^- λ *cI857* ΔBAM(Δ58.0–71.3%)ΔH1 Δ(*cro R A J b2*) *bio uvrB ilv*] (11) was used as the host for pLA2311 derivatives. Plasmid pTG801 (13) was provided by P. Silverman. The transfer proficiency and pilus-specific phage sensitivity of all cultures used for Western blot (immunoblot) analyses were tested and confirmed to be the expected phenotype.

Inner membrane preparations. Initially, inner membrane fractions were prepared by the procedure of Laine et al. (23) modified to accomodate larger sample volumes. Subsequently, we found a procedure adapted from Ito et al. (20) to be equally effective and more convenient. In this case, cells from a 30-ml LB medium (27) late-log-phase culture were collected, washed in 10 ml of 30 mM Tris buffer (pH 8.0), and suspended in 1 ml of Tris-EDTA (100 μM Tris, 0.5 mM EDTA [pH 8.0]) containing 20% sucrose. Lysozyme (final concentration, 100 μg/ml) was added, and after 30 min at 0°C, spheroplasts were disrupted by sonication. DNase (final concentration, 10 μg/ml) was added, the mixture was diluted with an equal volume of Tris-EDTA, and 2 ml was layered on top of a step gradient containing 3 ml of 53% and 7 ml of 15% (wt/vol) sucrose in Tris-EDTA. After 4 h of centrifugation at 60,000 rpm in a Beckman 70.1 Ti rotor, a 2-ml inner membrane fraction was collected from the 53%–15% sucrose interface.

Prior to use a 1- to 2-ml inner membrane fraction was diluted with Tris-EDTA buffer and spun at 50,000 rpm in a Ti 70.1 rotor at 6°C for about 10 min. Proteins in the pellet were then separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), as described previously (23, 37), with 10 to 16% exponential gradient gels, 10 W constant power, and a Bio-Rad model 221 or Protean II gel apparatus. The amount of pilin in any given membrane preparation could be estimated by examining a silver-stained SDS-polyacrylamide gel. Typically, for Western blots, inner membrane pellets were suspended in 50 μl of sample buffer and boiled for 3 min, and 40-μl samples were analyzed.

Detection of F pilin on Western blots. Antiserum reactions with pilin polypeptides were tested by a Western blot procedure. To enrich pilin and reduce interference from other proteins, we elected to use inner membrane fractions for analysis of membrane pilin reactions. All samples were first fractionated by SDS-PAGE as described above. Proteins were then transferred to 0.22-μm-pore-size nitrocellulose filters either in a TransPhor (Hoeffer Scientific Instruments) or Bio-Rad TransBlot SD (semidry) transfer apparatus. The 0.22-μm pore size was helpful because of the small size of the pilin protein. After transfer, the filters were allowed to react with the primary antibody (JEL92 or JEL93) for at least 18 h. In all experiments shown, antibody complexes were detected with Auroprobe BL, and silver enhancement was with IntenSE as suggested by the manufacturer (Janssen Life Sciences), except that incubation with the secondary antibody was also for at least 18 h.

RESULTS

F pili contain both 7- and 8-kDa polypeptides. Interestingly, preliminary analyses of silver-stained SDS-polyacrylamide gels showed that two polypeptide bands could be resolved from preparations of purified, wild-type F pili (Fig. 1A, lane 1), suggesting that two subunit types were present in these filaments. The dominant species migrated at 7 kDa, as expected for the mature F-pilin subunit. The second band migrated more slowly at approximately 8 kDa, in a position resembling that of the *traA* product which Laine et al. had named Ap8 (23). Inner membrane samples from an F*lac* host also contained these 7- and 8-kDa bands (Fig. 1A, lane 2). On Western blots reacted with a polyclonal anti-pilus serum, both bands were also detected in samples containing 5 to 100 μg of purified F pili or inner membranes prepared from an F*lac* strain (Fig. 1B). However, when the polyclonal serum was used, background reactions with other membrane components frequently made it difficult to identify pilin unambiguously.

The 7- and 8-kDa polypeptides both react with JEL93 and JEL92. Western blots with the monoclonal antibodies JEL93 and JEL92 provided a much more specific assay. As shown in Fig. 2A, JEL93 reacted well with the two polypeptide bands in samples of purified F pili and with corresponding bands present in inner membranes from an F*lac* strain. JEL93 did not give a positive reaction with any proteins in inner membranes from an F*lac traA1* mutant derivative or from the F^- host (JC3272), indicating that synthesis of the 7- and 8-kDa polypeptides required expression of the F-pilin structural gene, *traA*. The same results were also obtained with JEL92 (data not shown). Both JEL93 and JEL92 could also react with the two 7- and 8-kDa polypeptides in inner membranes from cells carrying pOX38, a Tra^+ F derivative

TABLE 2. JEL92 and JEL93 reaction with inner membrane pilin polypeptides expressed by F derivatives carrying *tra* and *trb* gene mutations

Gene	F*lac* mutant tested[a]	*kan* insertion mutant tested[b]	Reference	Pilus expression	Reaction with:	
					JEL92	JEL93
traM	F*lac traM102*[c,d]		1	+	+	+
traJ	F*lac traJ26*[c]		46	−	−	−
traY		F*lac traY244*[e]	21	−	−	−
traA	F*lac traA1*		46	−	−	−
traL	F*lac traL311*[c,d]		45	−	+	+
traE	F*lac traE7*		46	−	+	+
traK	F*lac traK105*[c]		46	−	+	+
traB	F*lac traB2*		46	−	+	+
traP		pOX38-*traP474*	21	NT[f]	+	+
trbD		pOX38-*trbD405*	15	NT	+	+
trbG		pOX38-*trbG476*	21	NT	+	+
traV	F*lac traV569*		34	−	+	+
traR		pOX38-*traR354*	24	+	+	+
traC	F*lac traC5*		46	+	+	+
trbI		pOX38-*trbI472*	29	+	+	+
traW	F*lac traW546*		33	+	+	+
traU	F*lac traU526*		33	(+)[f]	+	+
trbC		pOX38-*trbC460*	28	−	+	+
traN	F*lac traN548*		33	+	+	+
trbE		pOX38-*trbE453*	27	+	+	+
traF	F*lac traF43*		46	−	+	+
trbA		pOX38-*trbA236*	22	+	+	+
traQ		F*lac traQ238*	22	−	−	−
trbB		pOX38-*trbB242*	22	+	+	+
traH	F*lac traH55*[c]		46	−	+	+
traG	F*lac traG24*		46	−	+	+
	F*lac traG81*		46	−	+	+
	F*lac traG86*		46	−	+	+
	F*lac traG42*[c]		46	+	+	+
	F*lac traG79*		46	+	+	+
traD	F*lac traD8*		46	+	+	+
trbH		pOX38-*trbH290*	24	+	+	+
traI	F*lac traI41*[c]		46	+	+	+
	F*lac traI566*		33	+	+	+

[a] Except as noted, the F*lac tra* defect is an amber mutation and strain JC3272 was the host.
[b] All *kan* insertion mutants were tested in host XK1200.
[c] *traM102* and *traK105* are frameshift mutations; *traL311* is a UGA nonsense mutation; *traJ26*, *traH55*, *traG42*, and *traI41* are not suppressible.
[d] Tested in host ED2149.
[e] This *traY* mutation affects expression of additional *tra* genes (24).
[f] NT, not tested; (+), reduced numbers of pili are expressed (36).

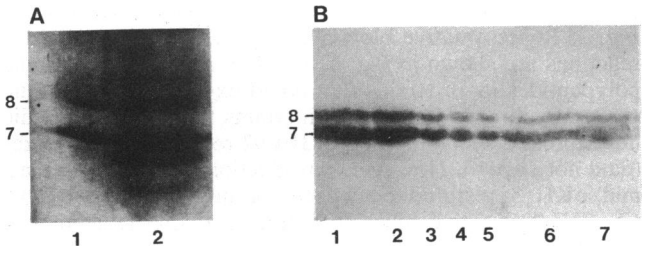

FIG. 1. Detection of pilin in purified filaments and inner membranes. (A) Bottom portion of gel showing proteins in preparations of purified F pili (lane 1) and inner membranes from an F*lac* strain (lane 2) after fractionation by SDS-PAGE and silver stain. (B) Western blot of a similar gel after reaction with polyclonal anti-F-pilus serum and immunogold detection. Lanes 1 to 5 contained, respectively, 100, 60, 20, 10, and 5 μg of purified F pili; lanes 6 and 7 contained, respectively, 1 and 0.5 ml of an inner membrane fraction from JC3272/F*lac*. Numbers at the left indicate the positions of pilin polypeptides of about 8 and 7 kDa.

made by circularizing the large F *Hin*dIII fragment (Fig. 2B, lanes 1 and 4). Thus, cells carrying F*lac* or pOX38 appeared to express two (7- and 8-kDa) pilin polypeptides that included not only the internal pilin epitope recognized by JEL92 but also the acetylated amino-terminal sequence required for JEL93 recognition.

Specificities of JEL93 and JEL92 Western blot reactions. That our Western blot assay was sensitive to the difference in JEL93 and JEL92 specificities is also demonstrated in Fig. 2B. Lanes 2 and 3 compare the reactions of these sera with inner membrane samples from cells carrying the two compatible plasmids pKI301 and pKI150. pKI301 expresses *traA* products very efficiently after induction of the λp_L promoter on this plasmid (23), and pKI150 expresses the *traQ* product constitutively. Although both 7- and 8-kDa *traA* products are expressed from pKI301 in $TraQ^+$ cells (23), only JEL92 could react with these two bands (Fig. 2B, lane 2). JEL92 also detected the small quantity of unprocessed, 13-kDa *traA* product seen under these expression conditions (we did not detect this precursor in F*lac* or pOX38 hosts). JEL93 did not react at all with any *traA*-related product in the inner membrane sample prepared from the pKI301/pKI150 host.

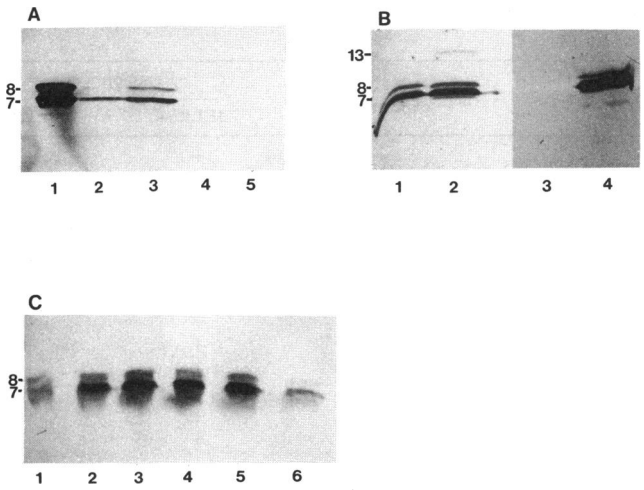

FIG. 2. JEL92 and JEL93 detection of pilin. Western blots, as for Fig. 1B, except monoclonal sera were used. (A) JEL93 reaction with pilin in 10 μg of purified F pili (lane 1) or inner membranes from a JC3272 host carrying F*lac* (lane 2), F*lac traG81* (lane 3), F*lac traA1* (lane 4), or no plasmid (lane 5). (B) JEL92 (lanes 1 and 2) and JEL93 (lanes 3 and 4) reactions with pilin in inner membranes from XK1200/pOX38 (lanes 1 and 4) or from the TraA$^+$ TraQ$^+$ strain XK1200/pKI301/pKI150 (lanes 2 and 3). (C) JEL93 reaction with pilin in inner membrane preparations from hosts carrying F*lac* (lane 1), F*lac traG24* (lane 2), F*lac traG42* (lane 3), F*lac traG79* (lane 4), F*lac traG86* (lane 5), and F*lac traL311* (lane 6).

These results confirmed that the JEL93 reaction, which requires amino-terminal acetylation of pilin, is dependent on an F gene product expressed by F*lac* and pOX38 but not by pKI301 or pKI150.

The data in Fig. 2B also suggest that the two 7- and 8-kDa *traA* products originally detected by Laine et al. (23) may have the same amino-terminal sequence. In cells that do not have the F gene product required for acetylation, these polypeptides react with JEL92 but not JEL93. However, in F*lac* or pOX38 hosts, both appear to become acetylated and to then have the complete amino-terminal epitope recognized by JEL93. We refer to the 7- and 8-kDa JEL93-reactive F-pilin polypeptides as Ac-7 and Ac-8, respectively. The corresponding pair of non-JEL93-reactive *traA* products expressed in TraQ$^+$ hosts which cannot acetylate pilin are referred to as *traA* products 7(Q) and 8(Q).

To discover which F gene products were required for acetylation of these proteins, we used the Western blot assay to test the reaction of JEL92 and JEL93 with membrane pilin polypeptides expressed by various F derivatives.

Analysis of F*lac* mutants carrying defects in *traG*. Since the pilin polypeptide expressed by phage EDλ134 reacted poorly with polyclonal anti-pilus serum, Laine et al. (23) suggested that acetylation of pilin subunits might require *traG*, the only F locus known to affect F piliation that was not expressed by the phage. Subsequent electron microscope analyses reported by Grossman et al. (12) supported this suggestion, since F-pilus filaments expressed by pTG801 hosts appeared to react with both JEL92 and JEL93. As this plasmid expresses a *tra* segment extending from the *Pst*I site in *traY* to the *Eco*RI site in *traG* (13), its products should differ from EDλ134 products only in including the TraG polypeptide required for piliation. Therefore, we assayed a full set of F*lac traG* amber mutants including derivatives carrying the mutations *traG42* and *traG79*, which result in transfer deficiency but do not affect F piliation, and derivatives carrying the mutations *traG24*, *traG81*, and *traG86*, which affect the N-terminal region of TraG and do prevent F-pilus assembly (31). Inner membranes of F*lac* strains carrying any one of these mutations reacted well with both JEL92 and JEL93 (Fig. 2A and C), and we concluded that TraG was not required for acetylation of F pilin. Firth and Skurray (6) reached a similar conclusion.

Analysis of F*lac* and pOX38 mutants carrying other *tra* gene defects. In additional tests, we assayed the JEL92 and JEL93 reaction with inner membrane proteins from a set of strains carrying F*lac tra* mutants defective in all of the other *tra* genes in which point mutations were available (Table 2). As expected, no pilin could be detected with either monoclonal antibody in inner membranes from strains carrying F*lac traA1* or F*lac traJ26*, since *traA* is the structural gene for F pilin and the *traJ* product is required for expression of most *tra* genes including *traA*. The pilin in all other inner membranes prepared from *tra* mutants in this set reacted well with both JEL92 and JEL93.

Nucleotide sequencing of *tra* region DNA had revealed a number of additional *tra* region loci (8, 19), and during the course of these studies, F and pOX38 derivatives that carry *kan* insertion mutations in many of these genes also became available. Table 2 also summarizes the results of JEL92 and JEL93 Western blot assays with inner membranes prepared from these strains. F pilin that was reactive with both antibodies was present in all cases but two: as expected, membranes from a *traQ* mutant failed to react with either JEL92 or JEL93, since this gene is essential for expression of the 7-kDa pilin polypeptide (22). Samples from the only F*lac traY* mutant strain available (F*lac traY244*) also did not react positively with either antibody; however, this mutation may affect *tra* operon expression (21). In all other cases, the intensity of the JEL93 reaction was sufficient to rule out an acetylation defect.

Analysis of Hfr deletion mutants. The F gene required for acetylation of F pilin was located by analysis of a series of Hfr deletion mutants that has previously been characterized by complementation analysis (16, 33). The progenitor of this series carried an F*lac* integrated into the *gal* locus on the host chromosome. The portion of the *tra* region that is still expressed in the deletion mutants we tested is diagrammed in Fig. 3. Deletions in the Tra$^+$ control strains, KI196 and KI529, extend only into *lacI* and *lacZ*, respectively, and do not affect the transfer region.

The Western blot reactions obtained with JEL93 and inner membrane proteins from these strains are also summarized in Fig. 3. Representative blots showing the JEL92 and JEL93 reactions are shown in Fig. 4. JEL92 reacted well with pilin polypeptides in all strains that could express both *traA* and *traQ*. Membranes from deletion strains that lacked *traQ* did not express detectable levels of JEL92-reactive *traA* products (data not shown). However, introduction of the TraQ$^+$ plasmid, pKI158, restored the capacity of these strains to synthesize JEL92-reactive pilin. In contrast, JEL93 reacted only with the pilin expressed by the control strains KI196 and KI529. It failed to react with the pilin expressed by any deletion mutant, including that in the TraD$^+$ TraI$^-$ mutant KI753. On well-resolved gels of radioactively labeled membrane protein preparations, the 7-kDa pilin polypeptide synthesized in the Hfr deletion strains also migrated slightly faster than the wild-type Ac-7 pilin subunit (data not shown). This trait is typical of the unmodified 7(Q) product synthesized when only *traA* and *traQ* are expressed (23, 49).

These results strongly suggested that the activity required to

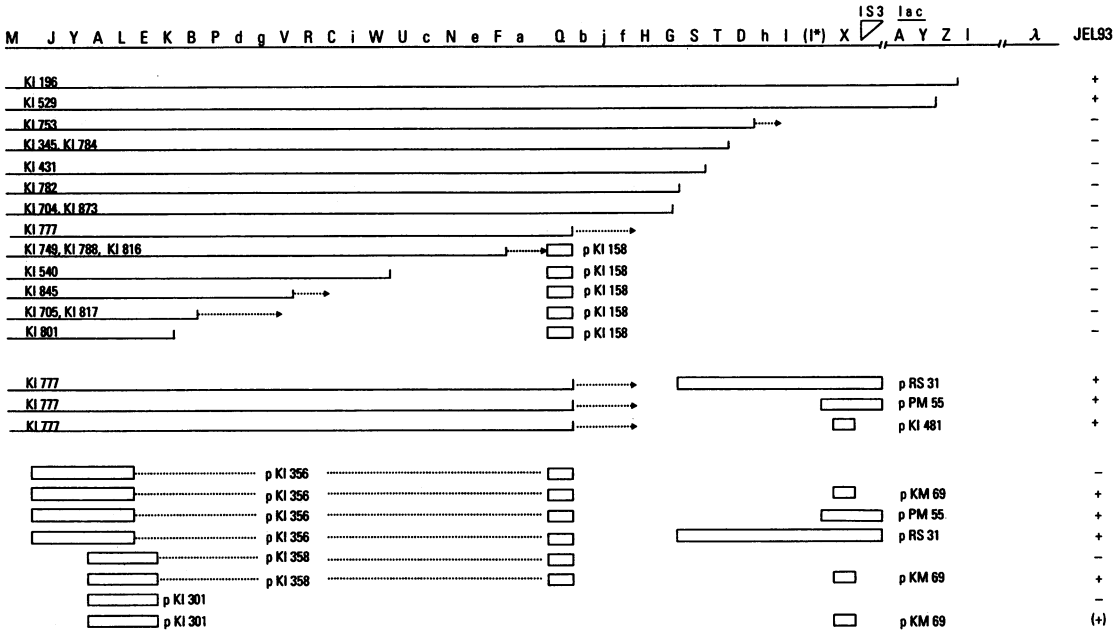

FIG. 3. Diagram of F transfer genes expressed by Hfr deletion mutants and various plasmids. A map at the top (not drawn to scale) indicates the relative position of F *tra* (capital letters) and *trb* (lowercase letters) genes, the IS*3a* insertion (in *finO*), *lac* genes, and λ prophage in the parental Hfr strain. Brackets show the *tra* genes remaining in the KI deletion strains listed (arrows show uncertainty of some endpoints). Bars show *tra* genes expressed from the plasmids indicated (pKI356 and pKI358 express two fragments). Strains carrying each set or combination of genes were analyzed. JEL92 reacted with pilin polypeptides in all strains diagrammed. + indicates that the JEL93 result was positive; − indicates that it was negative; (+) indicates a weak reaction (see Fig. 6).

acetylate the pilin polypeptide stemmed from the most promoter-distal segment of the *tra* region; that is, from a gene sequence located between *traI* and the IS*3* insertion in *finO*. These limits could be set because F*lac traI* amber mutants, a pOX38-*trbH*::*kan* mutant and pOX38 itself, all expressed pilin that reacted well with JEL93 (Table 2; Fig. 2B). Plasmid pOX38 lacks sequences distal to the *Hin*dIII site in IS*3a*.

Nucleotide sequencing has shown that the *traI*-to-IS*3* interval contains a 248-amino-acid open reading frame (*traX*) located just upstream from the IS*3*-interrupted *finO* se-

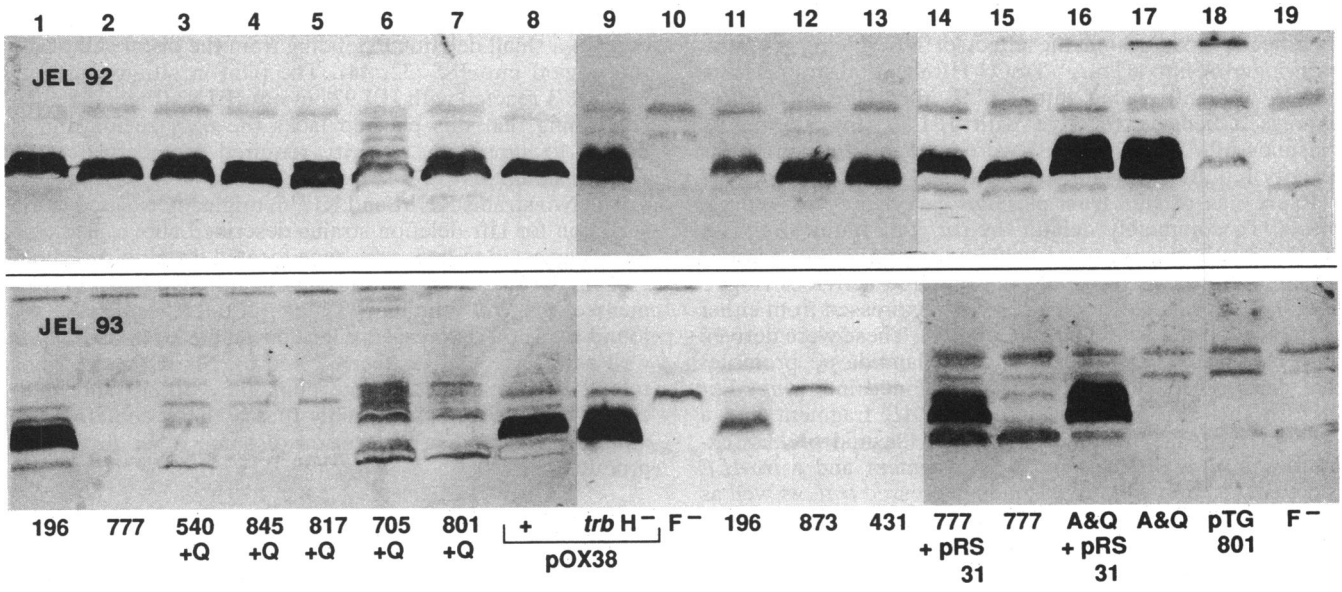

FIG. 4. Composite showing JEL92 (top panel) and JEL93 (bottom panel) reactions detected on Western blots. Numbers below lanes 1 to 7 and 11 to 15 correspond with the KI deletion strain tested; +Q indicates the strain also carried pKI158. In other lanes, strains tested were XK1200/pOX38 (lane 8), XK1200/pOX38-*trbH290* (lane 9), JC3272 (F^-) (lane 10), KI777/pRS31 (lane 14), XK1200/pKI356/pRS31 (lane 16); XK1200/pKI356 (lane 17); a pTG801 host (lane 18), and JC3272 (F^-) (lane 19). The background reactions with bands migrating above and below the pilins result from excess silver enhancement and/or secondary antibody recognition of contaminating outer membrane proteins.

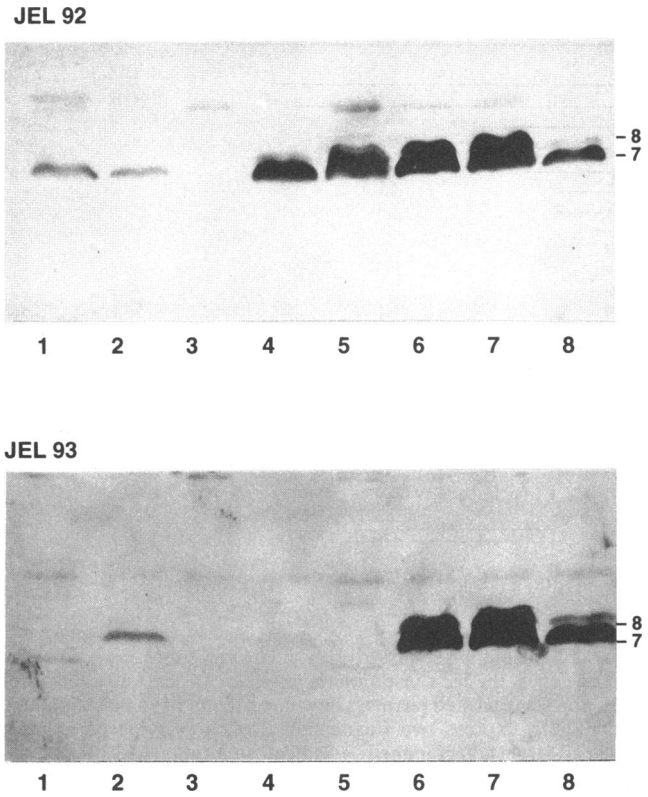

FIG. 5. The JEL92 (top panel) and JEL93 (bottom panel) reaction detected with inner membrane proteins from strains carrying pKI301 (TraA$^+$) (lanes 1), pKI301 and pKM69 (TraA$^+$X$^+$ (lanes 2), pKM69 (TraX$^+$) (lanes 3), pKI358 (TraA$^+$Q$^+$) (lanes 4), pKI356 (TraA$^+$Q$^+$) (lanes 5), pKI358 and pKM69 (TraA$^+$Q$^+$X$^+$) (lanes 6), pKI356 and pKM69 (TraA$^+$Q$^+$X$^+$) (lanes 7), and pKI356 and pRS31 (TraA$^+$Q$^+$X$^+$) (lanes 8).

quence (4, 51). The function of the *traX* gene has not been known. To confirm the importance of this locus in pilin modification, we tested the effect of introducing plasmids carrying *traX* into a TraA$^+$ TraQ$^+$ Hfr deletion strain. When pRS31 was introduced into KI777, the pilin polypeptide expressed became reactive with JEL93 (Fig. 3 and 4). Plasmids pPM55 and pKI481 were also able to express the activity required for synthesis of JEL93-reactive pilin.

Expression of pilin from plasmids carrying cloned *tra* segments. To completely delimit the *tra* gene requirement for F-pilin synthesis, we investigated the abilities of various plasmid constructs to supply the required activities (Fig. 3 and 5). The *traA* and *traQ* genes were expressed from either of the two plasmids pKI356 and pKI358. These were derived in vector pLa2311, which includes a lambda p_L promoter that can be used to express inserts cloned into *amp*. For pKI356 these inserts include a *traJYAL* fragment and a second fragment encoding only *traQ*. Plasmid pKI358 expresses inserts of the same *traQ* fragment and a *traALE* fragment. Although both plasmids expressed *traL* as well as *traA* and *traQ*, *traL* can not be essential to pilin synthesis since the *traL311* mutation had no effect on pilin expression (Table 2). Plasmid pKI301 contains the same *traALE* insert in pKI358 but does not contain a *traQ* gene. We expressed the *traX* sequence from a compatible plasmid, pKM69, which carries a small *Dsa*I-*Hin*dIII fragment containing the *traX* open reading frame but not the sequence encoding polypeptide TraI*.

After lambda p_L induction, membranes from cells carrying either of the TraA$^+$ TraQ$^+$ plasmids pKI356 or pKI358 contained pilin polypeptides that reacted with JEL92 but not JEL93 (Fig. 5, lanes 4 and 5). However, when the TraX$^+$ plasmid pKM69 was also expressed in the host, the pilin polypeptides became reactive with both JEL92 and JEL93 (Fig. 5, lanes 6 and 7). Therefore, we could conclude that the only F gene products required for synthesis of JEL93-reactive pilin are *traA*, *traQ*, and *traX* and that the product of *traX* is required for acetylation of the pilin polypeptide amino terminus.

Interestingly, we could detect some JEL93-reactive pilin in membranes from cells carrying pKI301 (*traALE*) and pKM69 (*traX*) (Fig. 5, lanes 2). As noted above, very little 7-kDa polypeptide is detectable when *traA* is expressed in the absence of *traQ* and only a faint JEL92-reactive band of this size could be seen when *traA* is expressed from pKI301 (compare the JEL92 reaction in Fig. 5, lane 1, with that in lane 4 or in Fig. 2B, lane 2). Nevertheless, at least some of this membrane pilin polypeptide was apparently processed and positioned appropriately for amino-terminal acetylation, since, in a TraX$^+$ host, it became reactive with JEL93.

Additional strains tested. We also characterized several additional strains for which the *traX* genotype or pilin acetylation phenotype was of interest.

Although electron micrographs had suggested that plasmid pTG801 expressed pilus filaments capable of binding JEL93 (12), this plasmid does not include *traX* (13). JEL93 did not give a positive reaction on Western blots of inner membrane proteins from an isopropyl-β-D-thiogalactopyranoside-induced pTG801 host, although JEL92 clearly did react with the pilin polypeptide in these samples (Fig. 4, lane 18). The amount of pilin present in inner membrane prepared from the pTG801 host was consistently low, possibly because so many pilus filaments are elaborated by this strain. Under our conditions, however, the reaction of wild-type pilin was typically stronger with JEL93 than with JEL92, and we concluded that pTG801 pilin polypeptide lacks the acetyl epitope required for a normal JEL93 reaction.

EDFL171 is a Tra$^-$ F*lac* plasmid derivative thought to contain a small deletion extending from the distal end of the *traI* region into IS*3* (32, 44). The pilin in strains carrying EDFL171 reacted with JEL92 but not JEL93 (Fig. 6, lane 1), confirming that this plasmid lacks the *traX* region and is unable to supply the activity required for acetylation of F-pilin subunits.

The two strains KI701 and KI736, originally isolated in the selection for Hfr deletion strains described above, had also been suggested to lack a *tra* gene located distal to *traI*; both exhibited a slight transfer deficiency, although they complemented the *traI* mutation tested (16). Nevertheless, we found that both expressed pilin that reacted with JEL93 as well as JEL92 (Fig. 6, lanes 2 and 3). Since these strains retain the ability to acetylate pilin, they do not appear to contain *traX* deletions; possibly they actually contain point mutations that affect expression of some other *tra* region product.

DISCUSSION

By exploiting the difference in specificity of the monoclonal antibodies JEL92 and JEL93, we have located the F gene required for amino-terminal acetylation of the F-pilin subunit. Although synthesis of a JEL92-reactive pilin polypeptide was dependent only on *traA* and *traQ*, the JEL93 reaction was additionally dependent on expression of

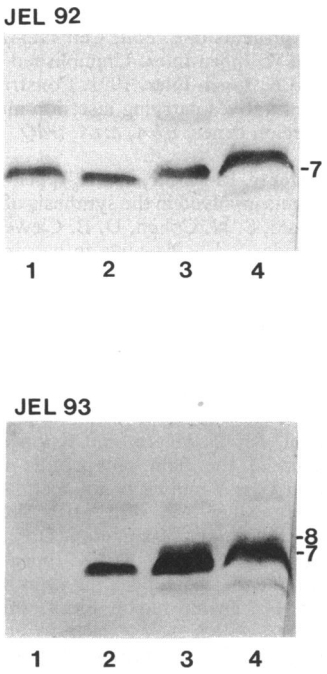

FIG. 6. The JEL92 (top panel) and JEL93 (bottom panel) reactions detected with inner membrane proteins from an EDFL171 strain (lanes 1), KI736 (lanes 2), KI701 (lanes 3), and KI196 (lanes 4).

the *traX* sequence. Since JEL93 recognizes the acetylated amino-terminal sequence of F pilin (9), we conclude that *traX* is required for acetylation of pilin. The fact that the pilin polypeptide synthesized by *traX* deletion strains migrated slightly faster than wild-type pilin was also consistent with this conclusion. In subsequent studies, pili purified from a *traX* mutant have been confirmed to contain subunits with an unblocked, Ala-Gly-Ser amino terminus (26).

The DNA sequence of the 248-codon *traX* open reading frame is highly conserved among F-like plasmids and predicted to encode a 27.5-kDa polypeptide containing several regions with inner membrane spanning potential (4, 51). Since the products of both *traX* and *traQ* appear to be inner membrane proteins (4, 48, 49), these proteins may normally be associated with each other. TraQ prevents degradation of the *traA* product and may assist membrane entry of the pilin precursor in a conformation appropriate for signal-peptide cleavage (23, 24). A closely associated *traX* product would then be positioned for catalyzing acetylation of the pilin polypeptide.

In contrast to eukaryotic systems, acetylation of bacterial proteins appears to be relatively rare. Only three *E. coli* genes that encode protein acetylation activities have been characterized. These, *rimI*, *rimJ*, and *rimL*, respectively encode small 161-, 194-, and 174-amino-acid products that catalyze amino-terminal acetylation of ribosomal proteins S18, S5, and L12 (43, 50). Computer comparison of the predicted *traX* product and sequences in the SwissProt data base did not reveal any remarkable stretches of amino acid homology to other acetylases that could unequivocally identify *traX* sequences involved in acetylation. Comparison with the short amino acid segments in the three *rim* products and an aminoglycoside acetylase that Tanaka et al. (43) had found to be similar did identify some *traX* product segments with similarity scores of 45 to 59% and 18 to 22% identity. However, these included hydrophobic amino acid sequences that might actually correspond to membrane spanning regions of TraX. Recent work also indicates that *traX* expresses two products which are both smaller than the 27-kDa product predicted from its sequence (25). Thus, additional information concerning the structure and conformation of TraX is needed for further evaluation of such data.

Interestingly, *traX* cannot be required for assembly of pilus filaments or for infection with pilus-specific bacteriophages. Hfr deletion strains that lack *traX* (e.g., KI753, KI345, KI431, KI782, and KI704) do express pili and are sensitive to both RNA and single-stranded DNA phages in the f2, Qβ, and f1 families (16). Strains carrying the F*lac* deletion plasmid EDFL171 are also sensitive to these phages (32). Since our data show that the pilin polypeptide synthesized by these strains is not acetylated, this modification is apparently dispensible in filament assembly.

Characterization of a recently constructed pOX38 *traX*::*kan* mutant indicates *traX* is also dispensible in transfer (26). Acetylation might enhance the stability of subunits and could be more critical under natural mating conditions. There is evidence that the structure of pili containing unacetylated subunits does differ somewhat from that of wild-type F pili. JEL92 and JEL93 do not bind along the length of wild-type F pili, indicating that the acetylated amino terminus of pilin is not exposed on the lateral surface of assembled filaments (9). However, Grossman et al. (12) found that JEL92 did decorate pili expressed by pTG801, unless pRS31 was also present to supply distal *tra* region gene activities. The unmasked amino-terminal residues in pTG801 pili can now be interpreted as a probable consequence of the more hydrophilic, unblocked, pilin polypeptide amino terminus; pRS31 expresses *traX* and would restore the hydrophobic, acetylated form. The similar, but less extensive, interaction observed between JEL93 and pTG801 pilus filaments (12) may represent a background reaction or a residual level of interaction between JEL93 and N-terminal pilin amino acid residues that could be detected in the electron microscope assay.

Our data show not only that synthesis of wild-type F pilin requires expression of the *traA*, *traQ*, and *traX* sequences but also that these three genes express the only F products required to make normal pilin subunits. The 7-kDa inner membrane polypeptide (Ac-7) expressed from plasmids carrying *traA*, *traQ*, and *traX* comigrated with, and appeared immunologically identical to, the membrane pilin and F-pilus subunits expressed by a wild-type F plasmid. However, it is very intriguing that our gels revealed a second type of pilin subunit (Ac-8) in both pili and membranes from wild-type cells. Plasmids expressing only *traA* and *traQ* similarly synthesized two JEL92-reactive products: the pilin polypeptide [7(Q)] and a second pilin band [8(Q)] that migrated at a position of approximately 8 kDa. That the pair of bands became reactive with JEL93 when *traX* was additionally expressed in the host suggests that both 7(Q) and 8(Q) can be acetylated and that the acetylated products (Ac-7 and Ac-8) have the same acetylated amino-terminal sequence. Thus, 8(Q) seems unlikely to be a processing intermediate that retains part of the TraA signal sequence. Further studies suggest that the 8-kDa form of pilin is derived by modifying the 7-kDa pilin polypeptide (24). As both the Ac-8 and Ac-7 forms of F pilin are present in preparations of purified F pili, the subunits in these filaments may be more heterogeneous than previously thought. This raises additional questions concerning the nature of the modification and the contribu-

tion of the modified subunits to pilus structure and conjugative transfer.

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REFERENCES

1. **Achtman, M., R. A. Skurray, R. Thompson, R. Helmuth, S. Hall, L. Beutin, and A. J. Clark.** 1978. Assignment of *tra* cistrons to *Eco*RI fragments of F sex factor DNA. J. Bacteriol. **133:**1383–1392.
2. **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.
3. **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.
4. **Cram, D. S., S. Loh, K.-C. Cheah, and R. A. Skurray.** 1991. Sequence and conservation of genes at the distal end of the transfer region on plasmids F and R6-5. Gene **104:**85–90.
5. **Finlay, B. B., L. S. Frost, W. Paranchych, J. M. R. Parker, and R. S. Hodges.** 1985. Major antigenic determinants of F and ColB2 pili. J. Bacteriol. **163:**331–335.
6. **Firth, N., and R. Skurray.** 1992. Characterization of the F plasmid conjugation gene, *traG*: two products for two functions? Mol. Gen. Genet. **232:**145–153.
7. **Frost, L. S., B. B. Finlay, A. Opgenorth, W. Paranchych, and J. S. Lee.** 1985. Characterization and sequence analysis of pilin from F-like plasmids. J. Bacteriol. **164:**1238–1247.
8. **Frost, L. S., K. Ippen-Ihler, E. G. Minkley, Jr., and R. Skurray.** Unpublished data.
9. **Frost, L. S., J. S. Lee, D. G. Scraba, and W. Paranchych.** 1986. Two monoclonal antibodies specific for different epitopes within the amino-terminal region of F pilin. J. Bacteriol. **168:**192–198.
10. **Frost, L. S., W. Paranchych, and N. Willetts.** 1984. DNA sequence of the F *traALE* region that includes the gene for F-pilin. J. Bacteriol. **160:**395–401.
11. **Gottesman, M. E., S. Adhya, and A. Das.** 1980. Transcription antitermination by bacteriophage lambda N gene product. J. Mol. Biol. **140:**57–75.
12. **Grossman, T. H., L. S. Frost, and P. M. Silverman.** 1990. Structure and function of conjugative pili: monoclonal antibodies as probes for structural variants of F pili. J. Bacteriol. **172:**1174–1179.
13. **Grossman, T. H., and P. M. Silverman.** 1989. Structure and function of conjugative pili: inducible synthesis of functional F pili by *Escherichia coli* containing a *lac-tra* operon fusion. J. Bacteriol. **171:**650–656.
14. **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.
15. **Hamilton, C. M., and K. Ippen-Ihler.** Unpublished data.
16. **Ippen-Ihler, K., M. Achtman, and N. Willetts.** 1972. Deletion map of the *Escherichia coli* K-12 sex factor F: the order of eleven transfer cistrons. J. Bacteriol. **110:**857–863.
17. **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.
18. **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.
19. **Ippen-Ihler, K., and R. A. Skurray.** Genetic organization of transfer related determinants on the sex factor F and related plasmids. *In* D. Clewell (ed.), Bacterial conjugation, in press. Plenum Press, New York.
20. **Ito, K., T. Sato, and T. Yura.** 1977. Synthesis and assembly of the membrane proteins in *E. coli*. Cell **11:**551–559.
21. **Kathir, P., and K. Ippen-Ihler.** Unpublished data.
22. **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.
23. **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 Press, New York.
24. **Maneewannakul, K., S. Maneewannakul, and K. Ippen-Ihler.** Synthesis of F pilin. J. Bacteriol. **175:**1384–1391.
25. **Maneewannakul, K., S. Maneewannakul, and K. Ippen-Ihler.** 1992. Sequence alterations affecting F plasmid transfer gene expression: a conjugation system dependent on transcription by the RNA polymerase of phage T7. Mol. Microbiol. **6:**2961–2973.
26. **Maneewannakul, K., D. Moore, and K-Ippen-Ihler.** 1992. The amino terminus of the pilin polypeptide in pilus filaments produced by an F *traX* mutant is not acetylated, p. 194, H-67. Abstr. 92nd Gen. Meet. Am. Soc. Microbiol. 1992. American Society for Microbiology, Washington, D.C.
27. **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.
28. **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.
29. **Maneewannakul, S., K. Maneewannakul, and K. Ippen-Ihler.** 1992. Characterization, localization, and sequence of F transfer region products: the pilus assembly gene product, TraW, and a new product, TrbI. J. Bacteriol. **174:**5567–5574.
30. **Manning, P. A., B. Kusecek, G. Morelli, C. Fisseau, and M. Achtman.** 1982. Analysis of the promoter-distal region of the *tra* operon of the F sex factor of *Escherichia coli* K-12 encoded by *Eco*RI restriction fragments f17, f19, and f2. J. Bacteriol. **150:**76–88.
31. **Manning, P. A., G. Morelli, and M. Achtman.** 1981. *traG* protein of the F sex factor of *Escherichia coli* K-12 and its role in conjugation. Proc. Natl. Acad. Sci. USA **78:**7487–7491.
32. **McIntire, S., and N. Willetts.** 1978. Plasmid cointegrates of F*lac* and lambda prophage. J. Bacteriol. **134:**184–192.
33. **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.
34. **Mintz, Y., and K. Ippen-Ihler.** Unpublished data.
35. **Moore, D., and K. Ippen-Ihler.** Unpublished data.
36. **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.
37. **Moore, D., B. A. Sowa, and K. Ippen-Ihler.** 1981. Location of an F-pilin pool in the inner membrane. J. Bacteriol. **146:**251–259.
38. **Moore, D., B. A. Sowa, and K. Ippen-Ihler.** 1981. The effect of *tra* mutations on the synthesis of the F-pilin membrane polypeptide. Mol. Gen. Genet. **184:**260–264.
39. **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.
40. **Paranchych, W., and L. S. Frost.** 1988. The physiology and biochemistry of pili. Adv. Microbiol. Physiol. **29:**53–114.
41. **Remaut, E., P. Stanssens, and W. Fiers.** 1981. Plasmid vectors for high efficiency expression controlled by the P_L promoter of coliphage lambda. Gene **15:**81–93.
42. **Skurray, R. A., H. Nagaishi, and A. J. Clark.** 1976. Molecular cloning of DNA from F sex factor of *Escherichia coli*. Proc. Natl. Acad. Sci. USA **73:**64–68.
43. **Tanaka, S., Y. Matsushita, A. Yoshikawa, and K. Isono.** 1989. Cloning and molecular characterization of the gene *rimL* which encodes an enzyme acetylating ribosomal protein L12 of *Es-*

cherichia coli K12. Mol. Gen. Genet. **217:**289–293.
44. **Traxler, B. A., and E. G. Minkley, Jr.** 1987. Revised genetic map of the distal end of the F transfer operon: implications for DNA helicase I, nicking at *oriT*, and conjugal DNA transport. J. Bacteriol. **169:**3251–3259.
45. **Willetts, N.** 1973. Characterization of the F transfer cistron *traL*. Genet. Res. **21:**205–213.
46. **Willetts, N., and M. Achtman.** 1972. Genetic analysis of transfer by the *Escherichia coli* sex factor F, using P1 transductional complementation. J. Bacteriol. **110:**843–851.
47. **Willetts, N., and R. Skurray.** 1987. Structure and function of the F factor and mechanism of conjugation, p. 1110–1133. *In* J. L. Ingraham, K. B. Low, B. Magasanik, F. C. Neidhardt, M. Schaechter, and H. E. Umbarger (ed.), *Escherichia coli* and *Salmonella typhimurium*: cellular and molecular biology. American Society for Microbiology, Washington, D.C.
48. **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.
49. **Wu, J. H., D. Moore, T. Lee, and K. Ippen-Ihler.** 1987. Analysis of *Escherichia coli* K12 F transfer genes: *traQ*, *trbA*, and *trbB*. Plasmid **18:**54–69.
50. **Yoshikawa, A., S. Isono, A. Sheback, and K. Isono.** 1987. Cloning and nucleotide sequencing of the genes *rimI* and *rimJ* which encode enzymes acetylating ribosomal proteins S18 and S5 of *Escherichia coli* K12. Mol. Gen. Genet. **209:**481–488.
51. **Yoshioka, Y., Y. Fujita, and E. Ohtsubo.** 1990. Nucleotide sequence of the promoter-distal region of the *tra* operon of plasmid R100, including *traI* (DNA helicase I) and *traD* genes. J. Mol. Biol. **214:**39–53.