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Characterization of *traX*, the F Plasmid Locus Required for Acetylation of F-Pilin Subunits

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

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

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Acetylation of F-pilin subunits has previously been shown to depend upon expression of the F plasmid transfer operon gene *traX*. To assess the requirement for pilin acetylation in conjugative transfer of F, we constructed *traX*::*kan* insertion mutations and crossed them onto the transmissible F derivative pOX38. Under standard conditions, the function of *traX* seemed to be dispensable. Although pilin synthesized by mutant plasmids pOX38-*traX482* and pOX38-*traX483* was not acetylated, F-pilus production and F-pilus-specific phage infection appeared to be normal and transfer occurred at wild-type frequency. Analysis of labeled products showed that TraX$^+$ plasmids expressed two approximately 24- (TraX1) and 22-kDa (TraX2) polypeptides that localized in the cytoplasmic membranes of cells. No product that was similar in size to the product predicted from the *traX* open reading frame (27.5 kDa) was detected. Therefore, we used site-directed mutagenesis, stop codon linker insertions, and *phoA* fusion analysis to investigate *traX* expression. Both TraX1 and TraX2 appeared to be encoded by the *traX* open reading frame. Insertion of a stop codon linker into the *traX* C-terminal coding region led to synthesis of two correspondingly truncated products, and fusions to *phoA* indicated that only the *traX* reading frame was translated. Expression was also very dependent on the *traX* M1 start codon; when this was altered, no protein products were observed. However, pilin acetylation activity was still detectable, indicating that some other in-frame start codon(s) can also be used. All sequences that are essential for activity are contained between *traX* codons 29 and 225. Sequence analysis indicated that *traX* mRNA is capable of forming a variety of base-paired structures. We suggest that *traX* expression is translationally controlled and that F-pilin acetylation activity may be regulated by physiological conditions in cells.

F pili are important components in the conjugative DNA transfer mediated by *Escherichia coli* plasmid F. The first step in conjugation is thought to occur when a donor F pilus attaches to a recipient cell; no conjugative DNA processes are initiated in F mutants that are unable to express these filaments. The F-pilus subunit, F pilin, is the product of the F *traA* gene (30), which encodes a 121-amino-acid polypeptide, propilin (11). The N-terminal residue of mature pilin corresponds to propilin residue Ala-52 and is acetylated (11). Maturation of the precursor depends upon the participation of two other F *tra* products. When the F *traQ* gene product is present, the *traA* product is efficiently translocated into the cytoplasmic membrane and its 51-amino-acid, amino-terminal signal peptide is removed (20, 23, 42). N-terminal acetylation of the pilin polypeptide requires an additional product that is expressed by the F *traX* gene sequence (31).

The existence of an F *tra* gene that is required for pilin acetylation was first predicted when pilin polypeptides that had been expressed from some TraA$^+$ TraQ$^+$ constructs did not react well with F-pilus antisera unless an F$^+$ host was used (20). The acetylated amino terminus is known to be the major F-pilin antigen and to account for up to 80% of the antibodies that are directed against purified F pili (9). Consequently, two monoclonal antibodies that had been raised against F pili provided a useful assay for F-pilin acetylation. One, JEL93, was found to recognize an acetylated octapeptide that corresponds to the N-terminal sequence of mature pilin; the other, JEL92, was found to be specific for an internal amino acid sequence that is centered around the ninth amino acid (methionine) of F pilin (10). Immunoanalysis of pilin polypeptides in inner membranes from Hfr deletion mutants demonstrated that synthesis of JEL93-reactive pilin depended on expression of the most distal F *tra* operon region, and it was shown that expression of the *traA*, *traQ*, and *traX* sequences was both necessary and sufficient for synthesis of mature, JEL93-reactive, F-pilin subunits (23, 31).

Although the *traX* locus was identified by DNA sequencing (6), little information about its product has been available. The properties of certain deletion mutants and the finding that F-like plasmid *traX* sequences are highly conserved suggested that this gene might be essential for conjugative transfer (6, 40, 43). However, all previously available *traX* mutants have been deletions which also lacked some adjacent *tra* DNA sequences. In addition, although F *traX* had been predicted to encode a 27.5-kDa, 248-amino-acid polypeptide, the F *traX* product had not been identified. Therefore, we constructed and characterized the transfer-associated properties of an F derivative that carried a *traX* mutation. We also characterized the polypeptide products expressed from various *traX* region DNA fragments and used stop codon linker insertions, site-directed mutagenesis, and protein fusions to delimit the sequence that is essential for pilin acetylation activity.

MATERIALS AND METHODS

Bacterial plasmids and strains. The construction of plasmids that expressed F *tra* region genes is summarized in Table 1. Vector pKI487 is similar to pSPORT1

* Corresponding author. Present address: Department of Biotechnology, Burapha University, Bangsaen, Chonburi 20131, Thailand.
† Deceased.

TABLE 1. Plasmids constructed for this study

Plasmid	Construction
pKI481	3.2-kb *tra Acc*I-*Hin*dIII fragment cloned into the pKI487 *Sma*I site; *traX* is in the T7 promoter orientation
pKI482	1.2-kb *Hin*cII pUC4K fragment cloned into the pKI481 *Dra*II site; *kan* is in the T7 promoter orientation
pKI483	1.2-kb *Hin*cII pUC4K fragment cloned into the pKI481 *Dra*II site; *kan* orientation is opposite to that of *traX*
pKM64	pKI481 with *Kpn*I-*Dsa*I deleted, removing the 5′ sequence of the *tra* fragment
pKM65	*Xba*I (amber codon) linker[a] inserted into the *Msc*I site on pKM64
pKM66	*Xba*I (amber codon) linker inserted into the *Rsr*II site on pKM64
pKM67	1.05-kb *tra Sma*I fragment cloned into the pKI487 *Sma*I site; *traX* is in the T7 promoter orientation
pKM70	pKM67 with a *Bsp*MI fragment deleted, removing the 5′ sequence of the *tra* fragment
pKM75	0.5-kb *traA Bcl*I-*Afl*II fragment cloned into the pKI487 *Sna*BI site; *traA* is in the T7 promoter orientation
pKM76	pKM67 with putative *traX* start codon (M1) altered from ATG to TCG
pKM78	pKM67 with a *kan Hin*dIII-*Sma*I fragment from pUC4KIXX (Pharmacia, Inc.) inserted at the pKM67 *Xba*I site; *kan* orientation is opposite to that of *traX*
pKM81	*lacI traX kan Pfl*MI-*Sna*BI fragment from pKM78 ligated with *Bam*HI-*Nhe*I-digested pKI158 (42); *traX* is under the control of the T7 promoter; *traQ* is under the control of the *amp* promoter and in the T7 promoter orientation; *kan* is oriented in opposition to *traX* and *traQ*
pKM85	0.83-kb *Sal*I fragment from pKM76 cloned into pKI487; *traX* is in the T7 promoter orientation
pKM91	pKM67 with *Kpn*I-*Msc*I deleted, removing the 5′ sequence of *traX*
pKM92	pKM67 with *Bsp*HI-*Sal*I deleted, removing the 3′ sequence of traX
pKM96	1.05-kb *tra Sma*I fragment cloned into pKSM717; *traX* is in the T7 promoter orientation
pKM99	3.5-kb *phoA*::*kan Bam*HI-*Eco*RV fragment from pSM2K cloned into the pKM67 *Bsp*HI site; *phoA* is in the *traX* orientation
pKM100	3.5-kb *phoA*::*kan Sma*I-*Eco*RV fragment from pSM2K cloned into the pKM67 *Bsp*HI site; *phoA* is in the *traX* orientation
pKM101	3.5-kb *phoA*::*kan SacI-Eco*RV fragment from pSM2K cloned into the pKM67 *Bsp*HI site; *phoA* is in *traX* orientation
pKM102	*Xba*I (amber codon) linker inserted into the pKM96 *Eco*RV site
pKM103	*Xba*I (amber codon) linker inserted into the pKM96 *Bsg*I site
pKM104	*traX Bst*EII-*Sac*I fragment from pKM96 inserted into the pSPORT1 *Sna*I site

[a] New England Biolabs, Inc., (Beverly, Mass.) linker no. 1062 [d(CTAGTCT AGACTAG)].

(GIBCO/BRL, Gaithersburg, Md.), except that *amp* is in the opposite orientation; it was isolated after digesting pSPORT1 DNA with *Bsp*HI, end filling, and religating. Plasmid pKI501 carries both a *lacUV5* promoter-regulated T7 RNA polymerase gene and a *lacI*^{q} repressor gene (22). Plasmid pKSM717 is a vector from which DNA inserts can be expressed from either a *lacUV5* or T7*lac* promoter (27); pSM2K carries a selectable *phoA-kan* cassette (28). Host strains, conjugation conditions, and other procedures that were used to obtain and characterize pOX38 *traX*::*kan* mutant recombinants were the same as those previously used to analyze the effects of *kan* insertion mutations in other F *tra* region genes (19, 21, 24–26).

Alkaline phosphatase activity in the *E. coli* K-12 strain CC118, (PhoA$^-$) on Luria-Bertani plates that contained 40 mg of 5-bromo-4-chloro-3-indolyl phosphate (XP) per ml. Luria-Bertani and other bacterial growth media, chemicals, and procedures for DNA manipulation have been described previously (24). Synthetic oligonucleotides and nucleotide sequence data were obtained from the Advanced DNA Technology Laboratory, Department of Biology, Texas A&M University. The Genetics Computer Group sequence analysis software package was used for computer analysis.

Protein analysis. Expression of DNA sequences under T7 promoter control was examined in hosts that carried a *lacUV5* promoter-regulated T7 RNA polymerase gene either on the chromosome (*E. coli* B strain XK100) or on plasmid pK501 (*E. coli* K-12 strains) (22). Conditions for IPTG (isopropyl-β-D-thiogalactopyranoside) induction of T7 RNA polymerase, rifampin treatment, and labeling (5 min) with [^{35}S]methionine have previously been described (22). After being labeled, cells were collected by centrifugation and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and autoradiography (24). Typically, the pellet from a 500-μl sample was suspended in 100 μl of SDS sample buffer and a 15-μl portion was fractionated by SDS-PAGE. Gene products expressed from other promoters were identified in maxicells as detailed previously (32).

Western blot (immunoblot) assays for pilin acetylation were performed essentially as described by Moore et al. (31), with monoclonal antibodies JEL92 and JEL93 (10). Bacteria were grown in 3 ml of Luria-Bertani medium to an optical density at 550 nm of 0.6 to 0.8; IPTG (final concentration, 1 mM) was added 1 h prior to sampling, when appropriate. Cells were collected from culture samples by centrifugation, suspended in SDS sample buffer, and fractionated by SDS-PAGE. After transfer to a 0.2-μm-pore-size nitrocellulose filter and incubation with monoclonal antibodies, JEL92 and JEL93 adsorption to pilin was detected with immunogold label.

Subcellular cytoplasmic, periplasmic, and membrane fractions were prepared as described by Wu et al. (41). Inner and outer membrane fractions were then separated on a sucrose step gradient that contained 2 ml of 70%, 4 ml of 53%, and 5 ml of 15% sucrose (24).

RESULTS

Construction of F derivatives defective in *traX*. To test the effects of a *traX* defect on transfer-associated properties, we first constructed insertion mutations in pKI481. This multicopy plasmid carries a 3.2-kb fragment from the distal end of the F *tra* region that extends from the *Acc*I site in F *traI* to the *Hin*dIII site (F coordinate 100/0) in the IS3 element that interrupts *finO* (Fig. 1). Mutations were then constructed by digestion with *Dra*II (*Eco*O109I) to remove part of *traX* and insertion of a *kan* gene cassette between the *traX Dra*II sites. In pKI482, *kan* and *traX* are transcribed in the same direction; in pKI483, *kan* is in anti-*tra* orientation (Fig. 1). Next, these mutations (*traX482* and *traX483*, respectively) were crossed onto plasmid pOX38, a transmissible F plasmid *Hin*dIII deletion derivative which retains an intact transfer region. The DNA of one recombinant plasmid that had been obtained from each cross was purified. Restriction analysis confirmed that these plasmids (pOX38-*traX482* and pOX38-*traX483*) carried the mutations we had constructed.

Characterization of pOX38-*traX482* and pOX38-*traX483*. Immunoblots of SDS-PAGE-fractionated proteins from the pOX38-*traX482* and pOX38-*traX483* mutant strains confirmed that these plasmids were defective in expression of *traX*-pilin acetylation activity. Both expressed pilin polypeptides that reacted normally with JEL92, which recognizes an internal F-pilin epitope (data not shown). However, these pilin polypeptides did not react with JEL93, which specifically recognizes the acetylated N-terminal F-pilin sequence (Fig. 2). After pKM67 had been introduced, pOX38-*traX* mutant strains became able to express JEL93-reactive pilin polypeptides (Fig. 2). As *traX* is the only F *tra* gene expressed by pKM67, these data confirmed that both pOX38-*traX482* and pOX38-*traX483* are defective in *traX*. They did not synthesize the mature, acetylated form of F pilin unless they had been complemented by expression of *traX* in *trans*.

Other properties of strains that carried pOX38-*traX* mutant derivatives are summarized in Table 2. Interestingly, *traX482* and *traX483* mutations had no appreciable effect on either transfer proficiency or F-pilus-specific phage sensitivity. Electron microscopy showed that pOX38-*traX* mutant cells produced pili that looked normal in character and number and to

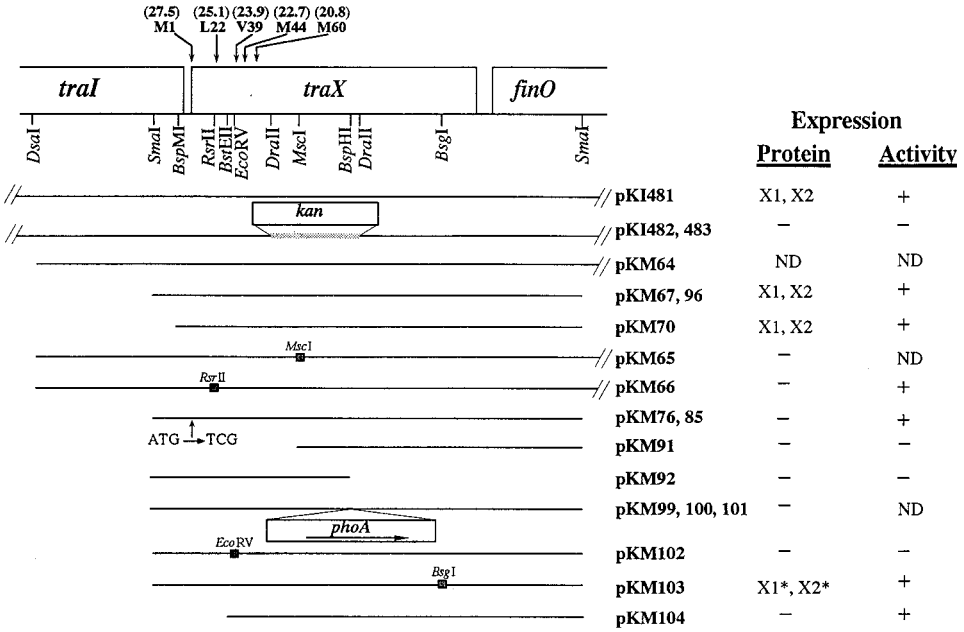

FIG. 1. Map of the F *traX* region. The position of *traX* is indicated by a closed box; portions of the adjacent *traI* and *finO* genes are shown as open boxes. The positions of restriction sites and potential start codons M1, L22, V39, M44, and M60 are also indicated; the predicted size of each product (in kilodaltons) is given in parentheses. The lines below indicate the DNA segment carried by the corresponding plasmid; slanted parallel lines indicate that the cloned segment extends beyond the *tra* region shown. A dotted line indicates a deletion, small square boxes indicate the positions of stop codon linker insertions and larger rectangular boxes indicate the positions of *kan* and *phoA* cassette insertions. X1 and X2, TraX1 and TraX2, respectively; X1* and X2*, truncated forms of TraX1 and TraX2, respectively; +, activity detected; −, no protein or activity detected; ND, not determined.

which R17 phages adsorbed. We have concluded that *traX* is dispensable under the standard conditions we tested and that unacetylated F-pilin subunits can be assembled into pili which are functional for both F-pilus-specific phage infection and conjugative transfer.

Detection and characterization of TraX1 and TraX2 polypeptides. Since the *traX* region can be expressed from the T7 promoter of pKI481 and its derivatives (Fig. 1), we examined proteins labeled in the presence of rifampin after induction of T7 RNA polymerase synthesis in hosts that carried these plasmids. Autoradiograms of SDS-PAGE-fractionated proteins which were labeled under these conditions are shown in Fig. 3. Although the F *traX* open reading frame (nucleotides 31938 to 32681 in the F *tra* region sequence [available under accession number U01159 in the GenBank database]) is predicted to encode a 27.5-kDa polypeptide (6), no labeled polypeptide that migrated to the 26- to 28-kDa region was detected in cells that carried pKI481. However, pKI481 did express products which migrated to positions that corresponded to approximately 24 and 22 kDa (TraX1 and TraX2, respectively) (Fig. 3A, lane 2). The same pair of polypeptide bands was labeled in cells that carried pKM67 (Fig. 3A, lane 4), which also expressed *traX* activity (Fig. 2). *traX*::*kan* insertion mutant derivatives pKI482 (Fig. 3A, lane 3) and pKI483 (data not shown) and the parental vector, pKI487 (Fig. 3A, lane 1), did not express these polypeptides. Since the absence of these two bands was the only observed difference to result from *traX482* and *traX483 kan* insertion mutations, we performed additional experiments to investigate the origin(s) of TraX1 and TraX2.

To test whether one or both of these products were the processed form(s) of a higher-molecular-weight precursor, cells that carried pKM67 were labeled with a short pulse (1

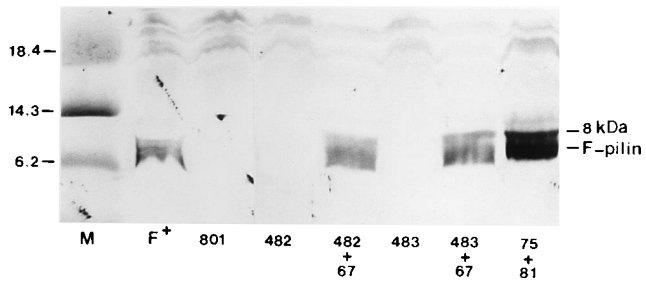

FIG. 2. JEL93 detection of acetylated F pilin. The positions of the two acetylated 7- (F-pilin) and 8-kDa pilin polypeptides are indicated on the Western blot shown. Samples were taken from whole cells that carried plasmids pOX38-*traX482* (482), pOX38-*traX482* and pKM67 (482 + 67), pOX38-*traX483* (483), and pOX38-*traX483* and pKM67 (483 + 67). Additional lanes contained control samples. Cells that carried pOX-Km (F^+) or pKM75 and pKM81 (75 + 81) expressed acetylated pilin polypeptides; cells that carried pTG801 (801) expressed unacetylated pilin (21). The sizes (in kilodaltons) and positions of prestained molecular weight markers (lane M) are also indicated on the left.

TABLE 2. Phenotypes of pOX38-*traX* mutants

Plasmid[a]	No. of transconjugants (per 100 donors)[b]	% Cells with pili	No. of pili/cell
pOX38-Km (Tra^+ control)	73	64	1.0
pOX38-*traX482*	78	59	0.97
pOX38-*traX483*	67	65	0.92

[a] In host strain VL584. With each plasmid tested, this strain was sensitive to F-pilus-specific RNA phages R17, Qβ, and f2 and filamentous DNA phages M13, f1, and fd.

[b] Donors and recipients (strain XK1200) were mixed at a 1:4 ratio and incubated at 37°C for 40 min prior to dilution and selection of Km^r Nal^r transconjugants.

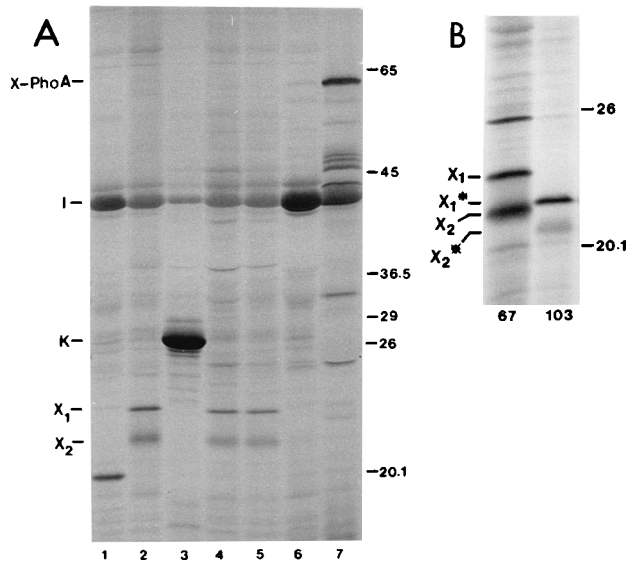

FIG. 3. Identification of *traX* products. Plasmid products were labeled with [^{35}S]methionine after T7 RNA polymerase induction and rifampin treatment and examined by autoradiography. (A) Lanes correspond to cells that carried pKI487 (1), pKI481 (2), pKI482 (3), pKM67 (4), pKM70 (5), pKM76 (6), or pKM99 (7). Cell samples were fractionated by SDS-PAGE with an exponential gradient (10 to 15%) of polyacrylamide. The positions of products expressed from *traX* (X1 and X2), *kan* (K), *lacI* (I), and the *traX*::*phoA* fusion on pKM99 (X-PhoA) are indicated on the left; the sizes (in kilodaltons) and positions of molecular weight markers are indicated on the right. (B) Conditions were the same as for panel A except that a 10% polyacrylamide gel was used and samples were taken from cells that carried pKM67 (67) and pKM103 (103). X1* and X2* indicate the truncated *traX* products expressed from pKM103.

min) of [^{35}S]methionine and sampled immediately and at intervals during a chase period of incubation with excess unlabeled methionine and chloramphenicol (Fig. 4A). Both polypeptide bands were detected at the end of the 1-min pulse, and neither showed an increase in intensity during the chase. Although the predicted product of *traX* was not expected to include a signal peptide sequence (6), we also tested the effects of ethanol on the synthesis of TraX polypeptides. We observed the same pattern of protein expression as we did in the absence of ethanol (data not shown). Thus, neither TraX1 nor TraX2 seemed to be derived from proteolytic processing.

As the *traX* open reading frame includes four cysteine codons, another possibility to be considered was that disulfide bonds cause the product to migrate aberrantly. Differences in disulfide bond formation are known to cause β-lactamase to run as a doublet unless a high-pH sample buffer is used to keep the protein in a fully reduced form (35). However, altering the pH of the sample buffer (pHs 2, 6.8, and 9 were tested) did not affect the positions or intensities of TraX1 and TraX2 bands (data not shown). Comparisons of samples that had been or had not been boiled in the presence or absence of reducing agent (β-mercaptoethanol) prior to SDS-PAGE also revealed no differences. Both TraX1 and TraX2 bands were present and consistently migrated to the same positions.

The subcellular location of TraX1 and TraX2 polypeptides. To examine the subcellular location of TraX1 and TraX2, maxicells that carried pKM64 were labeled and separated into cytoplasmic, periplasmic, and membrane fractions. Membranes were further fractionated on a sucrose step gradient which resolves inner and outer membrane components (16, 24). An autoradiograph of a gel on which each of these fractions was analyzed is shown in Fig. 4B. Unlike LacI and β-lactamase, which were primarily concentrated in the cytoplasmic and periplasmic fractions, respectively, both TraX1 and TraX2 were concentrated in the inner membrane fractions at the interface between 15 and 53% sucrose in the gradient (fractions 5 and 6). Outer membrane proteins, visualized by Coomassie blue staining, were concentrated at the interface of 53 and 70% sucrose (fractions 1 and 2; data not shown). Thus, both TraX1 and TraX2 appear to be inner membrane proteins.

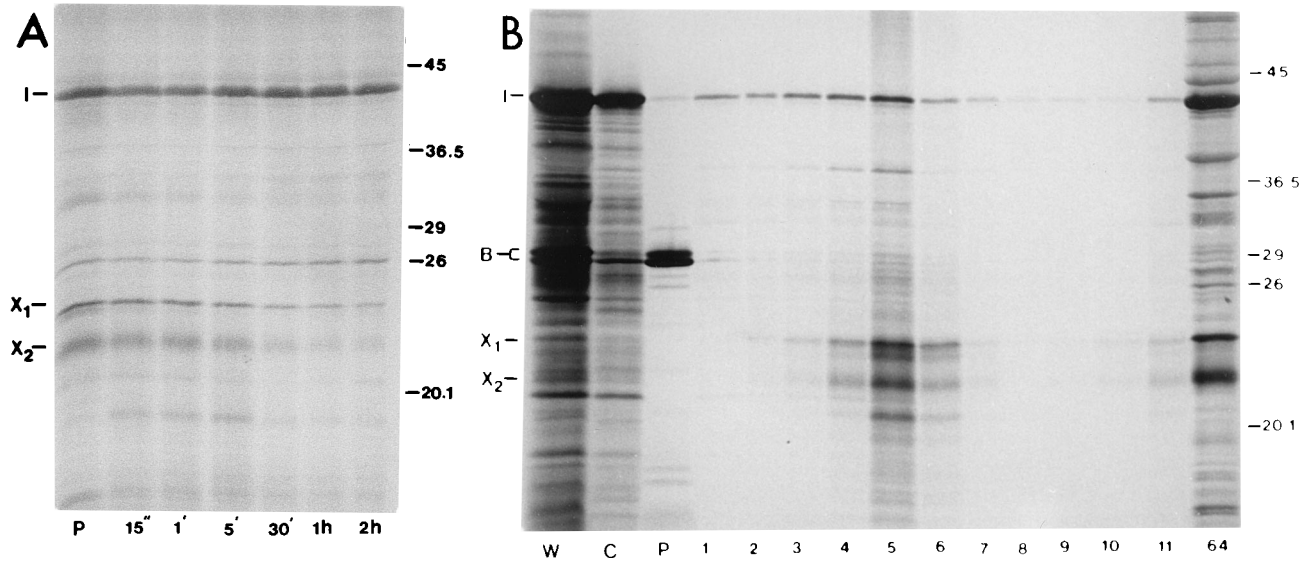

FIG. 4. Analysis of *traX* products. (A) Pulse-chase experiment. A culture of cells that carried pKM67 was labeled for 1 min and sampled immediately (P). Chloramphenicol and excess methionine were added, and additional samples were taken at the times indicated. (B) Subcellular localization. Maxicells that carried pKM64 were labeled and fractionated. The samples analyzed were whole cells (W), the cytoplasmic fraction (C), the periplasmic fraction (P), and membrane fractions of decreasing density (lanes 1 through 11). pKM64 (64) products labeled after T7 RNA polymerase induction of the host, XK100, are also shown. The positions of TraX1 (X1), TraX2 (X2), β-lactamase (B), and LacI (I) and of molecular weight markers (in kilodaltons) are indicated on the left and right, respectively.

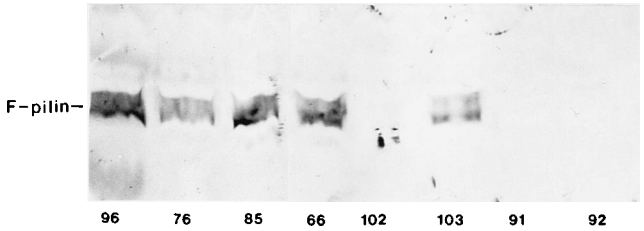

FIG. 5. F-pilin acetylation in pOX38-*traX482* derivatives that carried various pKM plasmids. The Western blot shown was reacted with JEL93. The number below each lane corresponds to that of the pKM plasmid tested (e.g., 96 corresponds to pKM96); the position of F pilin is marked.

Definition of sequences that are required for TraX1 and TraX2 expression. Since cells that carried pKM67 expressed both TraX activity (Fig. 2) and these two polypeptide bands, TraX1 and TraX2 (Fig. 3A, lane 4), all three features must derive from the 1,051-bp *Sma*I fragment that includes the open reading frame assigned to *traX* (744 nucleotides) and the 5′ portion of *finO* (Fig. 1). As no other open reading frame of an appropriate size was apparent in the reported sequence (6), we constructed an additional series of plasmids to test whether the difference between the predicted and observed sizes of *traX* region products is a result of the activities of unidentified translational start sites, reading frames, or termination sites. These data are discussed in the following sections and summarized in Fig. 1. Representative protein analyses and immunoblot data are shown in Fig. 3 and 5.

The effects of deletions on expression of TraX1 and TraX2. To remove potential start sites upstream from the M1 codon of the *traX* open reading frame, a *Bsp*MI fragment was deleted from pKM67 to obtain pKM70. This plasmid carries only 44 nucleotides of the *tra* DNA sequence that precedes the *traX* open reading frame, but it still expressed TraX1, TraX2, and *traX* F-pilin acetylation activity (Fig. 3A, lane 5). Subclones pKM91 and pKM92 did not express either of these TraX polypeptide bands (data not shown) and were unable to provide the activity required for pilin acetylation (Fig. 5). Thus, no *tra* sequence prior to the *Bsp*MI site was required for either TraX1 or TraX2 synthesis, and neither polypeptide appeared to derive solely from the upstream (prior to the *traX Bsp*HI site) or downstream (distal to the *traX Msc*I site) segment of *tra* DNA in our clones.

A stop codon insertion at *Bsg*I leads to truncation of both proteins. Insertion of a stop codon linker at the *Bsg*I site 70 nucleotides upstream from the termination codon for *traX* provided additional evidence that both polypeptides are encoded by the same DNA segment. pKM103 expressed a pair of shorter polypeptides, of approximately 22.6 and 21 kDa (X1* and X2*, respectively [Fig. 3B]). Thus, it appears that translation of both TraX1 and TraX2 normally proceeds through the *Bsg*I site and terminates at or near the same site. Interestingly, translation of the 23 *traX* codons that are distal to the *Bsg*I site is dispensable to F-pilin acetylation activity. After pKM103 had been introduced into the pOX38-*traX482* mutant strain, JEL93-reactive pilin was synthesized (Fig. 5).

Only one reading frame is translated across the *Bsp*HI site. We constructed three *phoA* fusions at the *Bsp*HI site near the center of the *traX* open reading frame to determine which DNA reading frames were translated in this region. The *phoA* cassette used does not include the *phoA* ribosome binding site, start codon, or signal peptide sequence (13), so *phoA* expression requires an in-frame fusion with a translational initiation site. Taking advantage of different restriction sites that flank the *phoA* cassette in pSM2K, we constructed fusions to all three of the forward reading frames in *tra* DNA (pKM99 [*Bam*HI; end filled]), pKM100 [*Sma*I], and pKM101 [*Sac*I; blunt]). None of these plasmids expressed TraX1 or TraX2, and only one (pKM99) expressed a fusion product (approximately 63 kDa; Fig. 3A, lane 7). No alkaline phosphatase activities were observed when pKM99 strains were plated on medium that contained XP. However, an immunoblot confirmed that the pKM99 fusion product reacted with anti-PhoA serum. DNA sequencing also confirmed that on pKM99, *phoA* is fused to the reading frame originally designated *traX*. We have concluded that the *traX* open reading frame is the only reading frame that crosses the *Bsp*HI site and is translated.

Alteration of the *traX* M1 start codon affects TraX1 and TraX2 synthesis. Additional experiments tested whether translation of TraX1 and/or TraX2 starts at a site that is distal to the M1 codon which begins the *traX* open reading frame. Figure 1 indicates the positions of some other codons in the *traX* open reading frame at which translation of appropriately sized products might be initiated. These include codons L22 (UUG; predicted product of 25.1 kDa), V39 (GUG; predicted product of 23.9 kDa), M44 (predicted product of 22.7 kDa), and M60 (predicted product of 20.8 kDa). A sequence with ribosome binding potential precedes each of these codons, but at an unfavorable distance (10 to 13 nucleotides).

We inserted a linker that contained stop codons in all three frames into several restriction sites located among these codons. We did not detect TraX1 or TraX2 synthesis when the products of plasmids that contained a stop codon linker insert at the *Rsr*II (pKM66), *Eco*RV (pKM102), or *Msc*I (pKM65) site were examined (data not shown). Since the linker insertion at the *Rsr*II site is only 47 nucleotides downstream from the M1 codon and M1 is the only potential translational start codon between the *Bsp*MI and *Rsr*II sites, expression of the TraX1 and TraX2 polypeptides appears to depend on the M1 start site. To confirm this, we used site-directed mutagenesis to alter the M1 AUG codon in pKM67 to the sequence TCG (a Ser codon). The resulting plasmid, pKM76, did not express detectable amounts of either TraX polypeptide (Fig. 3A, lane 6). Thus, synthesis of TraX1 and TraX2 appears to be highly dependent on M1-initiated translation.

Expression of *traX* activity is not completely dependent on translation initiated at the M1 codon. The pilin acetylation assay proved to be a more sensitive means of detecting expression. Interestingly, when we introduced our mutant plasmids into cells that carried pOX38-*traX482* and examined F-pilin acetylation, we found that some retained this *traX* activity despite the absence of detectable polypeptide synthesis (Fig. 5). JEL93 was able to react with pilin in the derivative that carried pKM76 (on which the M1 codon had been altered to TCG). The same result was obtained with a strain that carried pKM85, a subclone from pKM76 which lacks the ribosome binding sequence upstream from the (altered) M1 codon also. Acetylated pilin was also detected in a strain that carried pKM66, which contains the stop codon linker insertion at the *Rsr*II site prior to the L22 codon. Similarly, test strains that carried pKM104 also reacted with JEL93. In this construct, the amino-terminal coding segment of the *lacZ* sequence on vector pSPORT1 was fused, in frame, to the *traX* sequence at *Bst*EII so that a *lacZ*::*traX* fusion that lacked *traX* codons 1 through 28 was expressed. However, although pKM104 expressed pilin acetylation activity, we still did not detect a pKM104 *lacZ*::*traX* product when we examined labeled products that had been expressed from the *lac* promoter in maxicells or by the T7 polymerase expression system in XK100.

In contrast, a strain that carried pKM102, in which the linker

had been inserted at the *Eco*RV site, was unable to acetylate pilin, indicating that synthesis of an active product may depend either on translation through this site or (since the insert disrupted the putative ribosomal binding site for the V39 codon) on translational initiation at V39.

We can conclude that residues encoded by the *traX* sequence prior to *Bst*EII are dispensable to the pilin acetylation activity of the *traX* product(s). It is also clear that at least a low level of translation can be initiated at some secondary start codon(s) that is distal to the *Rsr*II site in the *traX* open reading frame. If translation from the M1 codon increases the efficiency with which the initiation site(s) is utilized, TraX1 and/or TraX2 may be products of such events.

DNA sequence analysis. Since no 27- to 28-kDa products were detected under any of the conditions tested, we also investigated the possibility of an error in the published sequence. We obtained and analyzed nucleotide sequence data for both strands of *traX* DNA. In particular, we looked for any difference that could cause the termination of translation initiated at the *traX* M1 codon but allow translation (re)initiated at more distal codons to proceed. The sequence we obtained was identical to the sequence that Cram et al. (6) reported.

DISCUSSION

The *traX* gene is of significant interest because its activity results in acetylation of the F-pilin amino terminus (31). Although deletions that extend through the *traX* sequence do not affect production of F-pilus filaments or F-pilus-specific phage infection (15), other previous results have suggested that *traX* is essential to transfer. Plasmid EDFL171, which carries a deletion that extends from IS3 through *traX* into *traI*, was found to be profoundly transfer deficient (29, 39), and the structure of pili expressed from a clone that lacks *traX* and other *tra* operon distal sequences has been shown to differ from that of normal pili (12). However, the pOX38-*traX* mutant plasmids we constructed were able to transfer with normal efficiency in standard mating tests. Since the pilin polypeptides that were synthesized in strains that carried these plasmids were not acetylated, it is clear that amino-terminal acetylation of this subunit is not essential for assembly of conjugally active pili.

Possibly, pilin acetylation is more critical to the assembly, stability, or function of pili in other hosts or under other physiological conditions. It is interesting that analysis of *Drosophila* cells indicates that either acetylated or nonacetylated cytoplasmic actin can participate in the assembly of microfilaments, but the latter is used less efficiently (5). There is evidence that the properties of pili produced by F *traX* mutants differ somewhat from those of wild-type F pili. We observed that cells that carried pOX38-*traX* mutants clumped together more frequently in liquid culture than did cells that carried pOX38-Km, suggesting that pili that contain unacetylated subunits are more adhesive than wild-type pili. We have also found that unacetylated pilin subunits are more likely to become modified to higher-molecular-weight forms (23). In addition, electron microscopy studies of pili expressed from a clone that lacks the *traD-traX* region indicate that the N-terminal regions of pilin subunits are more exposed along the lengths of such filaments than they are on wild-type pili (12).

Interestingly, though the F *traX* open reading frame is predicted to encode a 27.5-kDa polypeptide (6), we found that much smaller products (TraX1 and TraX2) were expressed by all of the $TraX^+$ plasmids we examined. These same polypeptides were detected when the products of pPD1 and pMP8, both of which contain larger *tra* segments (39), were labeled in maxicells (32). Similarly, the products of plasmid pOX38-*tra715*, which expresses the entire *tra* operon sequence, also include TraX1 and TraX2 (22). Thus, TraX1 and TraX2 seem to be typical products of *traX*.

Our data indicate that TraX1 and TraX2 differ in size from each other and from the predicted product because of the way their amino-terminal sequences are translated. We confirmed that no appropriately sized open reading frame other than *traX* is present in the sequence and found no evidence for post-translational product processing. As only one reading frame was translated across the *traX Bsp*I site and a stop codon linker insertion at the *traX Bsg*I site resulted in truncation of both protein products, TraX1 and TraX2 appear to end with the same C-terminal sequence.

However, our data also showed that the expression of both TraX1 and TraX2 is very dependent on the M1 codon at the beginning of the *traX* open reading frame. Although activity was expressed, we did not detect any labeled *traX* products when the M1 codon had been altered or stop codons had been introduced to block translation from this site. Nevertheless, we did not detect the full-length product expected from M1-initiated translation of the *traX* open reading frame. The most straightforward explanation of our data is that translation of the TraX1 and TraX2 polypeptides actually begins at another pair of start codons within the *traX* open reading frame and that translation from these sites is enhanced by translation initiated at the M1 codon. In this case, one (or all) of the codons in the cluster of V39, V42, and M44 presumably initiates translation of the polypeptides in one of the TraX bands we observed. An upstream (e.g., L22) or downstream (M60) codon might act as a start site for those in the other. The sizes of the polypeptide bands we observed (approximately 24 and 22 kDa) are consistent with several codon combinations (predicted products of 25.1 [L22], 23.9 [V39], 22.7 [M44], and 20.8 kDa [M60]).

At least one of the start codons that are distal to the *Rsr*II site in *traX* must be capable of initiating translation of this gene, since we found that F-pilin acetylation activity was still detected after the M1 codon had been changed from AUG to TCG and a stop codon linker had been inserted in the *Rsr*II site. As this activity was not expressed by pKM102, it might depend on low-level translation initiated at L22 or V39. It is not clear whether the polypeptides that were produced correspond to TraX1 and/or TraX2, since these *traX* products were not detected under these circumstances. Nevertheless, we can conclude that synthesis of an active product can be initiated at a codon(s) other than M1. This supports the hypothesis that the translation initiated at M1 regulates, but does not initiate, the synthesis of TraX1 and TraX2.

There are several ways that translation initiated at codon M1 might favor initiation at other *traX* start codons without yielding a detectable M1-initiated product. Studies with other transcripts have shown that sequence context, secondary structure, and other components can greatly influence the way in which RNA is translated. Specific translation factors can be involved, and large segments of transcripts can even be bypassed (3, 4, 14). The possibility that product proteolysis occurs is also not fully excluded. However, our data demonstrate that translation of sequences near the 5′ end of the *traX* open reading frame is dispensable to the activity of the product.

Our studies have also delimited the essential coding sequence. Both pKM104, which lacks *traX* sequences prior to the *Bst*EII site in *traX*, and pKM103, which contains a stop codon linker insertion at the *Bsg*I site, were able to express pilin acetylation activity. Therefore, all essential TraX protein domains must be encoded between these sites (codons 29 through

225 in the *traX* open reading frame). Interestingly, the subcellular location of TraX1 and TraX2, as well as sequence-based predictions, indicates that pilin acetylase is an inner membrane protein. The characteristics of the *traX*::*phoA* fusion protein we constructed further demonstrate that the residue that stems from codon 143 in the *traX* open reading frame is not on the periplasmic side of the cytoplasmic membrane. The fact that both propilin processing and acetylation occur so quickly during synthesis of F pilin (23) suggests that the requisite *traX* and *traQ* products ordinarily associate in the membrane and act in concert to effect translocation and maturation of F pilin.

N^{α}-acetylation of proteins has been detected most frequently in eukaryotic cells, and acetylation of bacterial proteins appears to be relatively rare. In *E. coli*, only three genes, *rimI*, *rimJ*, and *rimL*, are known to encode such enzymes. These products modify ribosomal proteins S18, S5, and L12, respectively (38, 43). Protein N^{α}-acetylases have generally been difficult to characterize because of their low cellular concentrations and extreme instability after purification (8). There is evidence, however, that acetylation can be regulated in response to particular growth conditions. Isozymes of yeast alcohol dehydrogenase have been found to exhibit variation in acetylation during different physiological conditions (18). Similarly, analysis of the ratio of L12 (unacetylated form) to L7 (acetylated form) polypeptides in *E. coli* ribosomal subunits has shown that L12 is the major form present during early stages of growth, but L7 becomes predominant in stationary phase (36). It has also been suggested that a general function for N^{α} acetylation is to protect proteins from proteolytic degradation (17). An *E. coli* mutant in which ribosomal protein S5 is not acetylated exhibits thermosensitivity (7), and the acetylated form of *Dictyostelium discoideum* cytoplasmic actin has been found to be more stable than its unacetylated form (37).

We conclude that expression of the F-pilin acetylation gene is influenced by translation of a dispensable part of the *traX* open reading frame. Although the precise mechanism involved requires further definition, it is interesting to consider why *traX* is regulated in such a complex posttranscriptional manner. One favorable result might be to regulate and reduce *traX* expression, relative to that of the upstream *tra* genes normally encoded on the same transcript. Certainly, the amounts of TraX1 and TraX2 we observed were small in comparison to those of other proteins we have expressed with the T7 polymerase system. The presence of a series of in-frame start codons together with the multiple products we observed further suggests a design that not only ensures *traX* expression but also allows the type as well as the quantity of the product to be varied. If activity depends on the amount of a particular product (e.g., TraX1 or TraX2) or on interaction that involves two products and host conditions determine the frequency at which different start sites are utilized, pilin acetylation may be coordinated with cellular physiology through variations in the ratio of different *traX* products. It will be of interest to test the effects of additional *traX* mutations and various translation conditions on the expression and activity of *traX*.

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