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Synthesis of F Pilin

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Transfer of the *Escherichia coli* fertility plasmid, F, is dependent on expression of F pili. Synthesis of F-pilin subunits is known to involve three F plasmid transfer (*tra*) region products: *traA* encodes the 13-kDa precursor protein, TraQ permits this to be processed to the 7-kDa pilin polypeptide, and TraX catalyzes acetylation of the pilin amino terminus. Using cloned *tra* sequences, we performed a series of pulse-chase experiments to investigate the effect of TraQ and TraX on the fate of the *traA* product. In $TraQ^-$ cells, the *traA* gene product was found to be very unstable. While *traA* polypeptides of various sizes were detected early in the chase period, almost all were degraded within 5 min. Rapid *traA* product degradation was also observed in $TraX^+$ cells, although an increased percentage of these products persisted during the chase. In $TraQ^+$ cells, most of the *traA* product was processed to the 7-kDa pilin polypeptide within the 1-min pulse period; this product [7(Q)] was not degraded but was increasingly converted to an 8-kDa form [8(Q)] as the chase continued, suggesting that host enzymes can modify the pilin polypeptide. Similar results were observed in $TraQ^+$ $TraX^+$ cells, but the primary 7-kDa product appeared to be N-acetylated pilin (Ac-7). An 8-kDa product (Ac-8) was also detected, but this band did not increase in intensity during the chase. We suggest a pathway in which TraQ prevents the *traA* product from folding to a readily degradable conformation and assists its entry into the membrane, Leader peptidase I cleaves the *traA* product signal sequence, and a subset of the pilin polypeptides becomes modified by host enzymes; TraX then acetylates the N termini of both the modified and unmodified pilin polypeptides.

Escherichia coli strains carrying the plasmid fertility factor, F, express specific, filamentous surface structures named F pili. These filaments have an essential role in plasmid transfer, initiating conjugal contact with recipient cells. Although a large number of the genes encoded by the F transfer region are required for F-pilus expression, only one polypeptide subunit, F pilin, has been detected in these filaments (24). Unassembled subunits have been found to be stored in the inner membrane of cells carrying the F plasmid (19). Earlier work has shown that the F *traA* gene encodes F pilin (8, 16) and that a second F gene, *traQ*, is also required for subunit synthesis (12, 21). Clones and transducing phages carrying *traA* expressed a polypeptide considerably larger than F pilin unless *traQ* was also expressed (11, 13). Analyses of the DNA sequence of *traA* and of F-pilin subunits confirmed that the *traA* product had to be processed; in mature pilin, a 51-amino-acid *traA* product signal sequence has been removed, and the amino terminus of the 70-amino-acid pilin polypeptide is acetylated (8).

The studies of Laine et al. (13) identified several *traA* products that appeared to be intermediates in F-pilin synthesis. In the absence of *traQ* expression, the major *traA* product detected was a polypeptide with an apparent molecular weight of 14,000, now presumed to correspond to the direct product of *traA* (13.2 kDa); minor quantities of a 7-kDa *traA* product could also be detected, but only when *traA* was expressed at a high level. In the presence of TraQ very little of the 13-kDa product was observed, and synthesis of a 7-kDa polypeptide [product 7(Q)] was very efficient. Concomitantly, a less intense 8-kDa polypeptide band was also detectable. However, neither of these products precipitated efficiently with polyclonal F-pilus antiserum unless an F^+ host was used, and Laine et al. (13) concluded that an additional F activity was required to acetylate F pilin.

Subsequently, the *traQ* locus was sequenced and shown to encode a 10.4-kDa membrane polypeptide (27, 28). Moreover, recent studies have demonstrated that *traX*, which also appears to encode a membrane protein (4), is the F gene required for acetylation of the amino terminus of F pilin (17). In the latter work, acetylated and unacetylated forms of pilin were distinguished by their reactions with monoclonal antibodies; JEL92 recognition depends on an epitope centered around Met-9 within the pilin sequence, whereas JEL93 recognition depends on the acetylated amino-terminal sequence (7). Moore et al. (17) also found that pilin subunits in inner membrane preparations or purified filaments resolved into two bands migrating at 7 and 8 kDa. Those from $TraA^+$ $TraQ^+$ $TraX^+$ hosts could both react with JEL93; thus, these appeared to have the same acetylated amino-terminal sequence and were referred to as Ac-7 and Ac-8. A similar pair of bands [7(Q) and 8(Q)] expressed by $TraA^+$ $TraQ^+$ $TraX^-$ hosts reacted only with JEL92. It was therefore of interest to reexamine the pathway for maturation of F pilin and to investigate the origin of the 8-kDa pilin polypeptides. In the experiments described here, we have used clones that express the *traA*, *traQ*, and *traX* genes and performed pulse-chase experiments to examine the fate of the *traA* product when it is expressed in the absence of other *tra* products or when TraQ and/or TraX is also present. On the basis of these results, we propose a pathway for F-pilin maturation.

MATERIALS AND METHODS

Bacterial strains and plasmids. Strain XK100 is a spectinomycin-resistant *E. coli* B host that carries a T7 RNA polymerase gene in its chromosome (14). For experiments performed in the *E. coli* K-12 host, SE5000 (F^- *araD139*

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TABLE 1. Plasmids

Plasmid	Description
pKI150	TraQ$^+$ Ap^r. 0.4-kb *Hin*cII-*Sca*I *traQ* fragment from pKI158 in pACYC177 *Sma*I site; *traQ* expressed from the *kan* promoter (17).
pKI158	TraQ$^+$ Km^r. 0.4-kb *Sau*3A-*Sma*I *traQ* fragment in pACYC177 *Hin*cII site; *traQ* expressed from the *amp* promoter (28).
pKM15	TraJ$^+$Y$^+$A$^+$L$^+$ Tc^r. 2.25-kb *traJYAL Hin*cII fragment cloned in pBR322 *Sca*I site and altered by replacing the *traY* promoter with the p_{T7} sequence (14).
pKM74	TraA$^+$X$^+$ Ap^r. 0.5-kb *Bcl*I-*Afl*II *traA* fragment and 1.7-kb *Dsa*I-*Hin*dIII *traX* fragment, regulated by the p_{T7} sequence on vector pKI487 (see Materials and Methods).
pKM75	TraA$^+$ Ap^r. Derived from pKM74 by deleting the 1.05-kb *Sma*I fragment that included *traX*.
pKM81	TraQ$^+$X$^+$ LacI$^+$ Km^r. Derived from pKI158 by deletion of *kan Bam*HI-*Nhe*I fragment and ligation with the *Pfl*MI-*Sna*BI fragment from pKM78. *traX* is expressed by p_{T7}; *traQ* is expressed by p_{T7} or the *amp* promoter; *kan* is in the opposite orientation (see Materials and Methods).
pOX38-*tra715*	Tra$^+$ Km^r. Derived by in vivo recombination of pKM15 and pOX38. The entire transfer operon is expressed from p_{T7}; after induction, plasmid transfer and F-pilus production occurs at the wild-type level (14).

lacΔU169 rpsL relA thi recA56) (22), plasmid pKI501, which contains a T7 RNA polymerase gene, was also present. In both cases, the T7 RNA polymerase gene is controlled by the *lacUV5* promoter. Other plasmids used in this study are described in Table 1.

Steps leading to construction of pKM74 were as follows: the 3.2-kb *tra Acc*I-*Hin*dIII region including *traX* was cloned in the *Sma*I site of pKI487 (14); in pKI481, *traX* is in the T7 promoter (p_{T7}) orientation. For pKM64, a *Kpn*I-*Dsa*I deletion was made to remove DNA at the 5′ end of the *tra* sequence (upstream from *traX*); for pKM74, a 0.5-kb *Bcl*I-*Afl*II *traA* fragment was then inserted at the *Sna*BI site. Steps used to construct pKM81 were as follows: the pKM64 *Sma*I fragment carrying *traX* was cloned in the *Sma*I site of pKI487; in pKM67, *traX* was in the p_{T7} orientation. A *kan* cassette (pUC4KIXX, *Hin*dIII-*Sma*I fragment) was inserted at the pKM67 *Xba*I site to obtain two plasmids with *kan* and *traX* in the same (pKM77) or opposing (pKM78) orientation. The *Pfl*MI-*Sna*BI fragment of these plasmids includes *lacI*, p_{T7}, *traX*, and *kan*. The *Pfl*MI-*Sna*BI fragment from pKM78 was ligated with pKI158, after deletion of the pKI158 *kan* sequence with *Bam*HI and *Nhe*I.

Pulse-chase experiments. Cells carrying plasmids were grown to an optical density at 550 nm of 0.5 at 37°C in JMM medium (15) containing antibiotics selective for those plasmids. For plasmid expression of genes controlled by a T7 promoter, isopropyl-β-D-thiogalactopyranoside (final concentration, 1 mM) was added to induce the expression of T7 RNA polymerase for 1 h before rifampin (final concentration, 100 μg/ml) was added. After an additional hour of incubation, the cells were labeled for 1 min with [^{35}S]methionine (10 μCi/1-ml culture). A 0.5-ml sample of cells was collected at the end of this pulse, and the rest of the culture was diluted with 2 volumes of JMM medium containing 1.2 mM unlabeled methionine and 26 μg of chloramphenicol per ml. A 1.5-ml sample of the culture was then collected at the chase time indicated. Each sample was microcentrifuged immediately for 3 min at 4°C. Cell pellets were resuspended with 100 μl of sodium dodecyl sulfate (SDS) sample buffer, and 15 μl was analyzed by SDS-polyacrylamide gel electrophoresis (PAGE) as described previously (15, 19, 20).

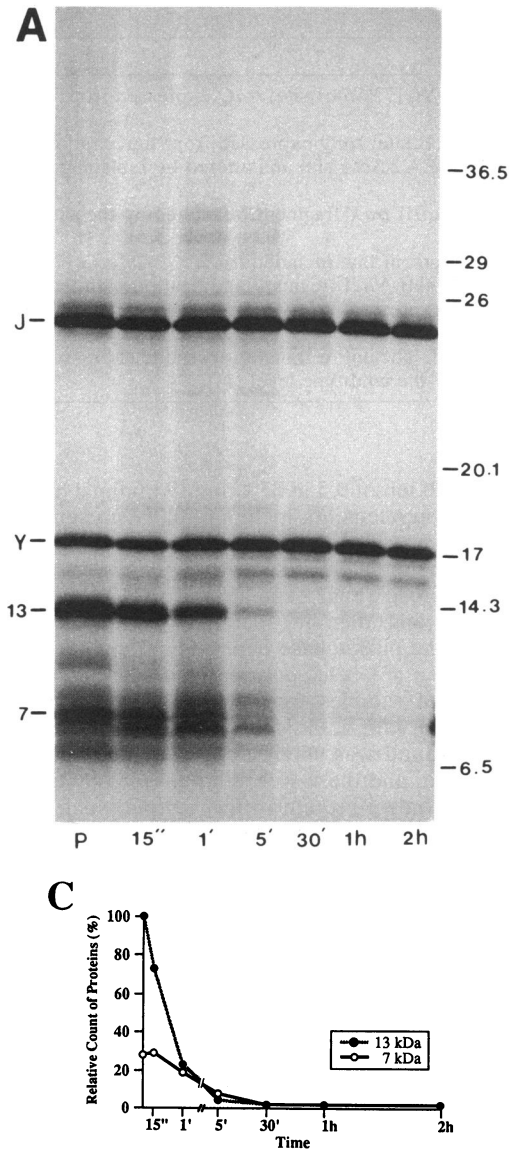
The effect of glucose was tested by growing the cells in JMM medium containing 0.2% glycerol instead of glucose; in this case, the culture was divided after the 1-min labeling period and incubated in either 0.2% glycerol medium or 0.2% glucose medium during the chase period. The possibility of osmoregulation was tested by the method of Inokuchi et al. (10). In this case, the cells were first grown to an optical density at 550 nm of 0.5 at 37°C in M9 minimal medium with appropriate supplements including 0.2% glycerol as a sole carbon source, centrifuged, washed in M9 minimal medium with the same supplements, resuspended in media containing 30% sucrose (high osmolarity) or 0.2% glycerol (low osmolarity), and then incubated at 37°C for 2 h before performing the pulse-chase experiment.

Quantitation of labeled polypeptide bands. SDS-polyacrylamide gels of samples from the pulse-chase experiments were scanned with a PhosphorImager (Molecular Dynamics). Background was subtracted from the values obtained for each band, and these were then divided by the value for an internal control protein in the sample, such as TraY or LacI. The data were plotted by using DeltaGraph version 1.5 on a Macintosh computer.

RESULTS

F-pilin synthesis was investigated by performing a series of pulse-chase experiments to examine the *traA* products detected in the presence and absence of TraQ and/or TraX activities. In all cases the *traA* gene was expressed from a T7 promoter after induction of T7 RNA polymerase and addition of rifampin. After plasmid products were labeled for 1 min with [^{35}S]methionine, unlabeled methionine (final concentration, 800 μM) and chloramphenicol (final concentration, 17 μg/ml) were added to inhibit further labeling and protein synthesis. Thereafter, samples were removed after various chase periods (15 s to 2 h), and the proteins were examined by SDS-PAGE and autoradiography.

***traA* products expressed in TraQ$^-$ TraX$^-$ cells.** To examine the *traA* products expressed in the absence of both TraQ and TraX, cells carrying either pKM15, which expresses a *traJYAL* fragment, or pKM75, which only expresses *traA*, were used in separate experiments. The results obtained in these pulse-chase experiments are shown in Fig. 1. In both cases, the intensity of the labeled *traA* product bands expressed by these plasmids decreased significantly during the chase, although quantitation of the TraJ and TraY bands expressed by pKM15 (Fig. 1A) or of the LacI product band expressed by pKM75 (Fig. 1B) showed that these were stable throughout the 2-h chase period. Immediately after the pulse period (Fig. 1A and B, lanes P), an intense 13-kDa band, presumed to be the direct product of *traA*, could be detected. In addition, at least six different, smaller, labeled polypeptides could already be detected in both the pKM15 and pKM75 samples; these included bands smaller and larger than that at



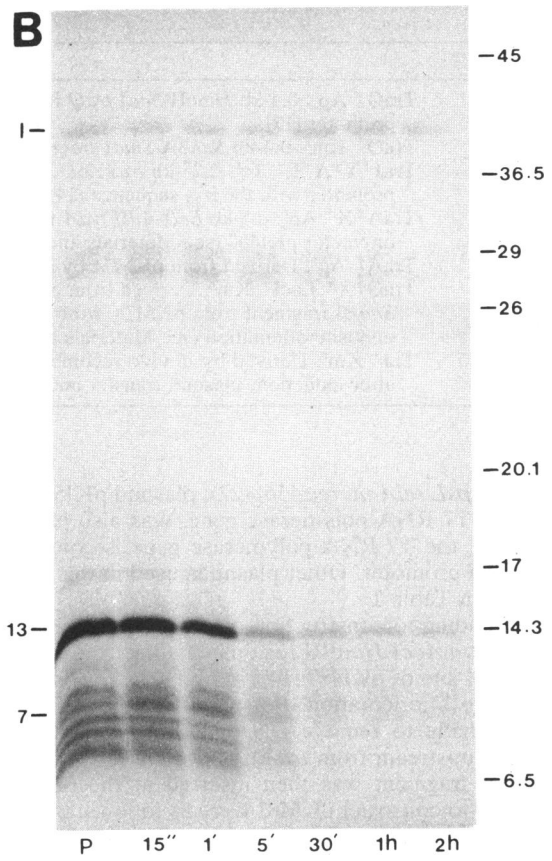


FIG. 1. *traA* products expressed in TraQ$^-$X$^-$ cells. Autoradiographs show SDS-PAGE-fractionated samples. (A) pKM15 (*traJYAL*) products labeled in XK100; (B) pKM75 (*traA*) products labeled in SE5000/pKI501. Products were labeled in the presence of rifampin for 1 min prior to addition of unlabeled methionine and chloramphenicol; samples were taken immediately after labeling (P) or after a 15-s; a 1-, 5-, or 30-min; or a 1- or 2-h chase period, as indicated. The positions of 13- and 7-kDa *traA* products, TraY (Y), TraJ (J), and LacI (I), and molecular mass marker proteins (sizes in kilodaltons) are also marked. (C) Relative counts present in 13- and 7-kDa bands in pKM15 samples as determined from the gel by a PhosphorImager; a set of parallel lines separates sampling times depicted on two different linear scales.

the 7-kDa position characteristic of F pilin. The relative amount of label associated with the 13- or 7-kDa product in samples of cells carrying pKM15 is plotted in Fig. 1C. Within a 15-s chase period, the amount of labeled 13-kDa product had dropped to about 72% of the level detected at the end of the pulse period, whereas the 7-kDa band seemed to remain at about the same level. After 1 min, the amount of 13-kDa product had decreased to about 22% of its original level and the amount of the 7-kDa product had also begun to decrease in intensity. By 5 min, the amount of both polypeptides had dropped dramatically, and after a prolonged chase period, the radioactivity in the 13- and 7-kDa bands, as well as all other *traA* bands, had decreased to a barely detectable level. Thus, under these conditions, the 13-kDa *traA* product appeared to be rapidly degraded by host proteins. None of the polypeptides derived from *traA* appeared to be stable.

The *traA* products formed in the presence of TraQ. To examine the fate of the *traA* product in the presence of TraQ, pKI150, a compatible plasmid carrying *traQ*, was introduced into the pKM15 strain used in the previous experiment. Both the autoradiograph and quantitative data from a pulse-chase experiment are shown in Fig. 2. Very little of the product was detectable as a 13-kDa band after the 1-min pulse-labeling period, and this amount had decreased further by the end of the chase period. However, a large amount of labeled 7-kDa polypeptide was already present by the end of the pulse period, and this band seemed to be quite stable throughout the 2-h chase. This 7-kDa polypeptide [7(Q)] corresponds to the product previously identified as the *traQ*-dependent processed product of *traA* (13, 28).

Interestingly, the intensity and definition of the 8(Q) band increased during the chase period. This *traA* product, which migrates at approximately 8 kDa, was also observed by Laine et al. (13) and originally suggested to be an intermediate of pilin processing or a by-product of TraQ activity. However, analysis of the relative count in the radioactive bands resolved in this experiment indicated that the intensity of the 8-kDa band increased concomitantly with a decrease in the intensity of 7(Q) (Fig. 2B). To test whether the 7(Q)

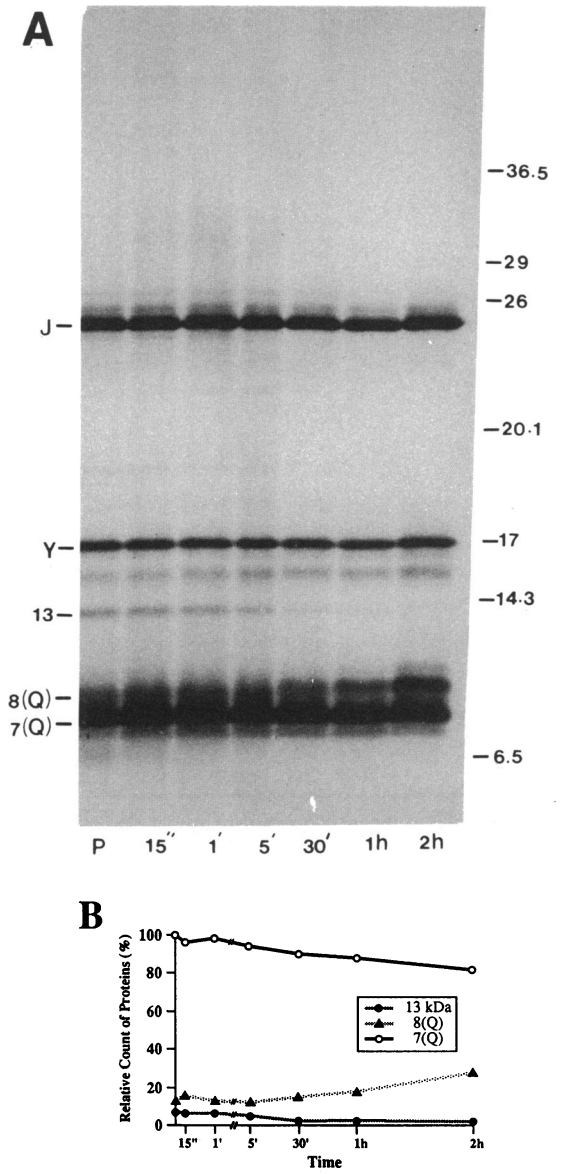


FIG. 2. *traA* products expressed in TraQ$^+$ cells. (A) Autoradiograph, as in Fig. 1, except pKM15 products were labeled in an XK100 host that carries the *traQ* plasmid pKI150; the 7- and 8-kDa *traA* products, 7(Q) and 8(Q), observed in TraQ$^+$ cells are indicated. (B) Relative counts in bands as determined with a PhosphorImager; a set of parallel lines separates sampling times depicted on two different linear scales.

product is a precursor of the 8(Q) polypeptide, we performed similar experiments using longer chase periods with two different strains (Fig. 3). One strain carried the pair of plasmids pKM75 (*traA*) and pKI158 (*traQ*), and the other carried pKM15 (*traJYAL*) and pKI150 (*traQ*). There was no significant difference between the results obtained with these different sets of plasmids. In both cases the prolonged chase led to a gradual decrease in the intensity of the 7(Q) polypeptide band and to a gradual increase in the intensity of the 8(Q) polypeptide band. We concluded that the 8(Q) product must be derived by covalent modification of the 7(Q) polypeptide. The data further suggested that there might be multiple modification events. At the earliest times the 8(Q) band was diffuse (Fig. 2A), possibly because modification occurred only at a subset of target sites; after a longer chase the 8(Q) band, presumably modified at all sites, was quite discrete. It seems most likely that host proteins accomplish this alteration. No F sequences other than *traA* and *traQ* are expressed by pKM75 and pKI158.

In other work, our laboratory has also found that preparations of purified pilus filaments and inner membranes from various F derivatives contain both 7- and 8-kDa pilin polypeptides (17). Since the ratio of the two polypeptides has been observed to vary in different preparations of purified pili (18), it seemed possible that the amount of 8-kDa product formed might depend on culture conditions. Using pulse-chase experiments as an assay system, we tested the effects of two environmental factors on the modification. The effect of glucose was investigated by growing and labeling cells carrying the *traA* and *traQ* plasmids (pKM75 and pKI158) or the *traJYAL* and *traQ* plasmids (pKM15 and pKI150) overnight in glycerol minimal medium. Immediately after the 1-min labeling period, these cultures were divided and chased in either glucose or glycerol minimal media. No difference between the samples was observed within a 3-h chase period in these media. In both cases, the intensity of the 7(Q) band decreased and that of the 8(Q) polypeptide increased during the chase (data not shown). Thus, the addition of glucose had no apparent effect on the modification reaction. Similarly, in an experiment in which the effects of high (30% sucrose)- and low (0% sucrose)-osmolarity media (10) were compared, we found no evidence that a difference in osmolarity affected the modification process (data not shown).

***traA* products expressed in the presence of TraQ and TraX.** The *traA* products detectable under TraQ$^+$ TraX$^+$ conditions were examined in a pulse-chase experiment with cells carrying the pair of plasmids pKM74 (*traA* and *traX*) and pKI158 (*traQ*). In general terms, the results of these pulse-chase experiments resembled those obtained for TraA$^+$ TraQ$^+$ cells (compare Fig. 4 with Fig. 2). Very little 13-kDa product was detectable at the end of the pulse, and this small amount became reduced during the chase period. Again, the majority of the label was associated with a 7-kDa pilin polypeptide, and this product seemed to be stable throughout the chase period. An 8-kDa product was also detected. In this case, however, we suggest that the 7- and 8-kDa products correspond to the acetylated pilin polypeptides Ac-7 and Ac-8. Western blots (immunoblots) have shown that the pair of pilin polypeptides expressed by TraA$^+$ TraQ$^+$ TraX$^+$ cells react with JEL93 (17). Although it is difficult to distinguish the acetylated and unacetylated forms of pilin in a pulse-labeling experiment, our data suggested that acetylation of pilin had occurred in these cells before the end of the 1-min pulse period; the 7-kDa product comigrated with mature pilin and in the slightly retarded position relative to 7(Q) that is typical of Ac-7 (Fig. 3 and 4).

The presence of TraX also appeared to alter the kinetics of expression of the 8-kDa protein. Although an indistinct 8-kDa band that remained stable could be detected in the earliest time periods, no further increase in the amount of this polypeptide was observed as the chase continued (Fig. 4). In an experiment in which the chase period was continued for 5 h (Fig. 3), it was also apparent that there was no significant increase in accumulation of the 8-kDa polypeptide. This suggested that, once the amino terminus of a 7-kDa pilin polypeptide has been acetylated, further modification to an 8-kDa form occurs either very slowly or not at all.

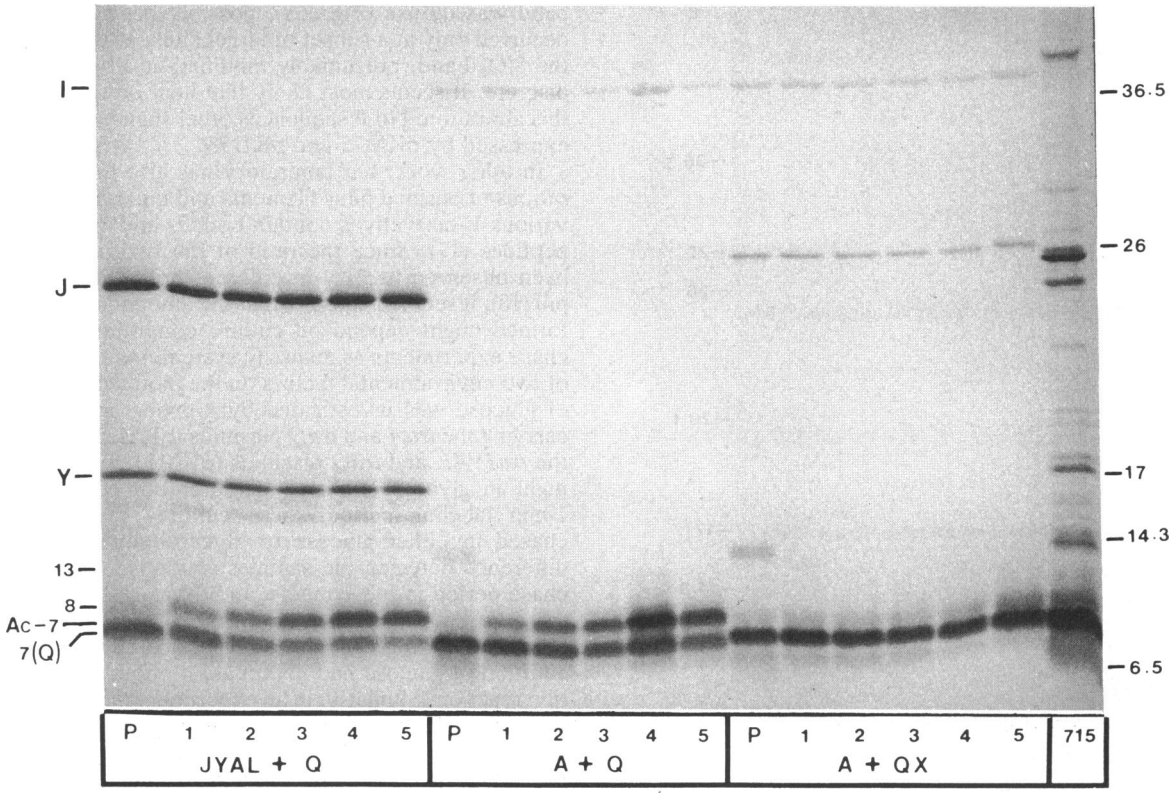


FIG. 3. *traA* products detected in TraQ$^+$ cells after a prolonged chase period. Products from *traA* plasmids were labeled for 1 min and sampled immediately (P) or after a 1- to 5-h chase. Samples were from XK100 strains carrying pKM15 and pKI150 (lanes JYAL + Q), pKM75 and pKI158 (lanes A + Q), pKM75 and pKM81 (lanes A + QX), or the transfer-proficient plasmid, pOX38-*tra715* (lane 715). The positions of the 13-kDa, 8-kDa, Ac-7, and 7(Q) *traA* products, TraY (Y), TraJ (J), LacI (I), and molecular mass (in kilodaltons) markers are indicated on the autoradiograph.

The *traA* products expressed in the presence of TraX. A pulse-chase experiment for *traA* products expressed together with TraX (but not TraQ) was performed in a strain carrying pKM74 which carries both the *traA* and the *traX* genes downstream from a T7 promoter (Fig. 5). At the end of the 1-min pulse period, the sample resembled that from cells expressing *traA* alone; the 13-kDa product was the major labeled polypeptide, and a series of other small polypeptides, including a 7-kDa product, was detected. Similarly, most of these *traA* polypeptides appeared to be unstable. After 1 min, the amount of 13-kDa product had decreased to about 60% of its original level, and within 5 min, it was only 20% of its original value. The band at 7 kDa decreased about 50% in intensity in the first minute and was reduced to 15% of its peak level within 5 min. A longer chase did not, however, result in further loss of radioactivity at the 7-kDa position. A larger portion of the 13-kDa polypeptide band also seemed to be more stable in these cells, since after 5 min, the rate of 13-kDa product disappearance slowed considerably. About 15% of the 13-kDa product could still be detected after 1 h, and this fraction appeared to remain stable. These data suggested that TraX can enhance the stability of some *traA* polypeptides. Nevertheless, it seemed that the stabilized 13-kDa products could not undergo signal peptide processing. In contrast, the portion of the 7-kDa product that remained after 5 min might be correctly processed and acetylated F pilin (Ac-7) (see below).

DISCUSSION

Our results support the pathway for F-pilin maturation proposed in Fig. 6. In this pathway, TraQ is required for efficient utilization and processing of the *traA* product to polypeptide 7(Q), and TraX catalyzes an amino-terminal acetylation reaction resulting in synthesis of Ac-7 (mature F pilin). Some 7(Q) polypeptides are modified to 8(Q), which has the same amino terminus but a higher apparent molecular weight. TraX-mediated N-terminal acetylation of 8(Q) yields Ac-8 F-pilin subunits. While we do not exclude the possibility that Ac-7 subunits can be modified to 8-kDa polypeptides, 7(Q) is suggested to be the more efficiently utilized substrate for this reaction. The ratio of 7- to 8-kDa subunits in pilus filaments will then vary if the efficiency of modification or acetylation changes under different physiological conditions. Although our data do not exclude other models, we also suggest that TraQ interactions prevent the *traA* product from folding into a readily degradable form and assist its entry into the membrane in a conformation appropriate for signal peptide processing.

Previous studies of F *traA* have examined *traA* products accumulated in maxicells or membrane preparations after labeling for longer periods or after reaction with anti-pilus sera (11, 13, 17, 27, 28). The primary (13-kDa) and minor (7-kDa) products detected in TraQ$^-$ cells were both enriched in inner membrane preparations, although their quantity was small compared with the amount of pilin expressed in TraQ$^+$

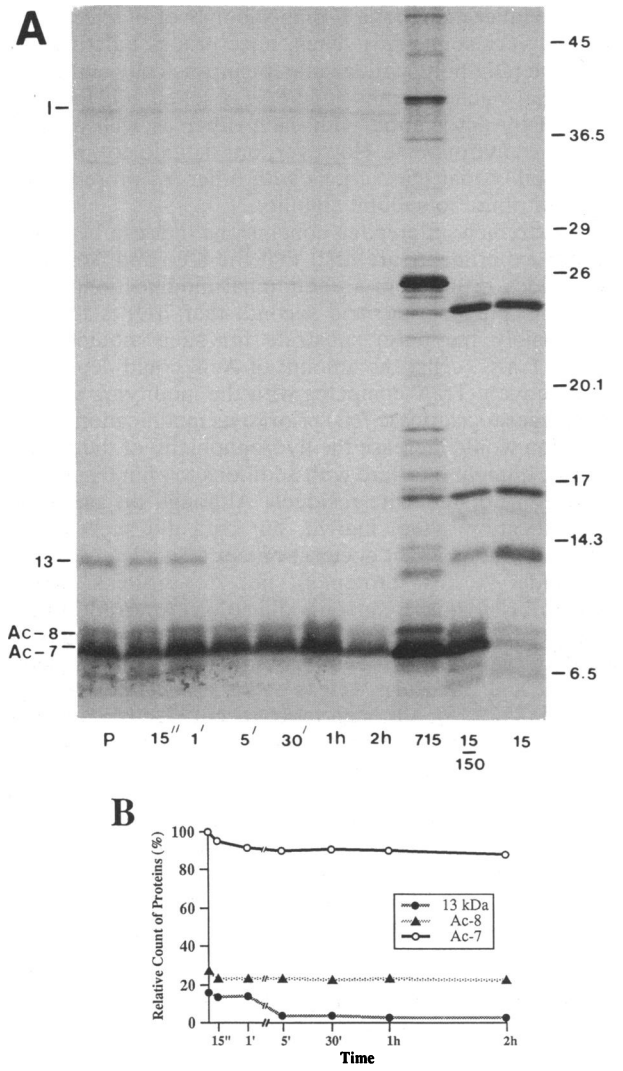


FIG. 4. *traA* products expressed in TraQ$^+$ TraX$^+$ cells. (A) Autoradiograph, as in Fig. 1, except pKM74 (*traA traX*) products were labeled in XK100 hosts that carried pKI158 (*traQ*). Additional lanes show labeled *tra* products expressed in cells carrying the Tra$^+$ plasmid, pOX38-*tra715* (lane 715); the *traJYAL* and *traQ* plasmids, pKM15 and pKI150 (lane 15/150); or pKM15 alone (lane 15). The positions of the 13-kDa, Ac-8, and Ac-7 *traA* products, LacI (I), and molecular mass (in kilodaltons) marker proteins are indicated. (B) Relative counts in bands as determined with a PhosphorImager; a set of parallel lines separates sampling times depicted on two different linear scales.

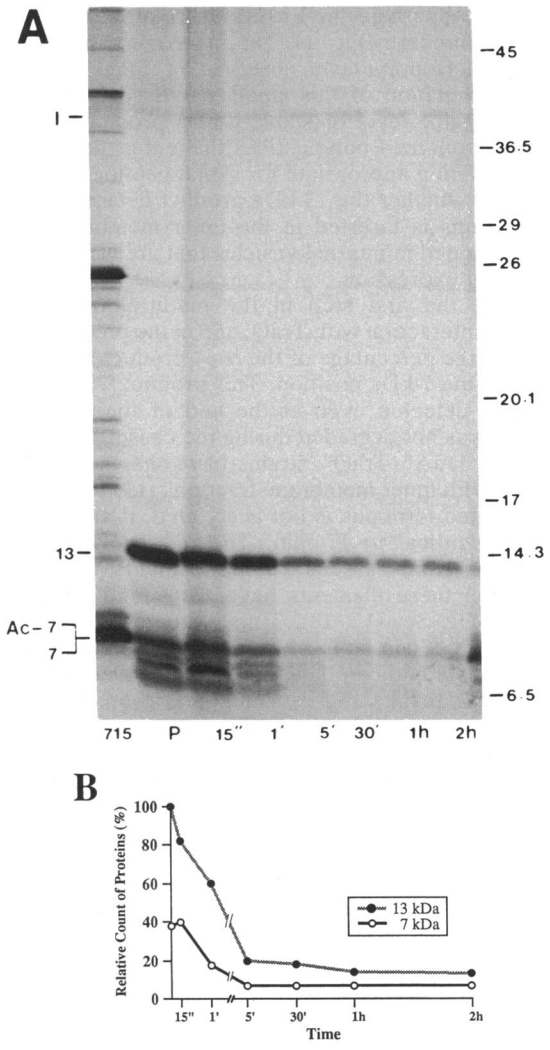


FIG. 5. *traA* products expressed with TraX. (A) Autoradiograph, as in Fig. 1, except pKM74 (*traA traX*) products were labeled in an SE5000/pKI501 host. A pOX38-*tra715* (lane 715) sample is also shown and the positions of the 13-kDa, Ac-7, and 7- and 8-kDa *traA* products, LacI (I), and molecular mass (in kilodaltons) marker proteins are indicated. (B) Relative counts in bands as determined with a PhosphorImager; a set of parallel lines separates sampling times depicted on two different linear scales.

cells. It now seems clear that this quantitative difference is attributable to the instability of the *traA* product in TraQ$^-$ cells rather than to a TraQ effect on the level of *traA* expression.

Most of the pulse-labeled 13-kDa *traA* product disappeared rapidly when *traA* was expressed in the absence of TraQ. At early times we detected additional *traA* polypeptides migrating at 10, 8, and 7 kDa, as well as some even smaller than pilin. However, the radioactivity in these bands also diminished very quickly, suggesting that most of these polypeptides were transient intermediates formed by proteolytic digestion. Thus, we suggest that almost all of the *traA* product made in TraQ$^-$ cells folds into a form that becomes degraded by cytoplasmic enzymes. Although degradation by periplasmic enzymes is also conceivable, we favor a model in which only a small fraction of the *traA* polypeptide associates with the cytoplasmic membrane under these

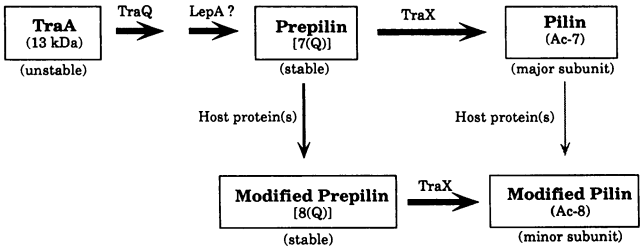


FIG. 6. The pathway for F-pilin synthesis.

circumstances; perhaps the 1 to 2% of the product remaining after a 30-min chase (Fig. 1). The inner membrane 13- and 7-kDa products previously observed in TraQ$^-$ cells may reflect accumulation of this small stabilized fraction. The barely detectable level of stable 7-kDa products observed could stem from *traA* polypeptides that enter the membrane in a conformation appropriate for signal peptide processing. It is unclear whether the 13-kDa product detected in membrane fractions is inserted in the inner membrane or, for example, trapped in internal vesicles that are artifacts of the conditions of expression.

Normally, the first step in the maturation of F pilin involves an interaction with TraQ, and in the presence of this protein, a large percentage of the *traA* product immediately appeared at the 7-kDa position. This product [7(Q)] was the major band detected even at the end of the 1-min pulse period and was not degraded during the chase. Other experiments with TraA$^+$ TraQ$^+$ strains have shown that 7(Q) is associated with inner membrane fractions (13, 17). Although the 7(Q) amino terminus is not acetylated, it appears to be otherwise identical to F pilin. The pilin polypeptides of TraX$^-$ strains can be assembled into filaments, and subunits purified from these filaments have the amino-terminal sequence Ala-Gly-Ser (17, 18). Thus, when TraQ is present, the *traA* product can be used efficiently, and the processed protein is stable and can be assembled into filaments.

All of these results suggest that TraQ protects the *traA* product from degradation and allows it to enter the membrane to be processed. A number of *E. coli* proteins have been shown to require "chaperone" proteins that keep the newly synthesized polypeptide in a conformation appropriate for translocation into the membrane (23). TraQ could function similarly to prevent pilin precursor from assuming a degradable conformation that cannot enter the membrane. However, TraQ is an inner membrane protein and could act by quickly channeling the *traA* product into the membrane and positioning it for processing and subsequent assembly. As previously suggested, removal of the signal sequence seems likely to be a function of the host leader peptidase, LepA (8, 13). Others in our laboratory are currently investigating the involvement of host secretory proteins and LepA in pilin synthesis.

If *traA* product degradation stems from folding of the protein into a protease-susceptible form, interactions with other proteins might also inhibit this degradation. Interestingly, the presence of TraX did appear to enhance the percentage of 13- and 7-kDa polypeptides that persisted during the chase period. TraX, like TraQ, is a cytoplasmic membrane protein and should also be able to interact with and, possibly, stabilize *traA* product sequences. TraX may even, ordinarily, be associated with TraQ and assist in positioning pilin in the membrane. Thus, association with TraX might slow folding of the *traA* product, permitting more membrane entry and LepA processing. Indeed, the portion of the 7-kDa polypeptide that persisted in TraA$^+$X$^+$ cells appeared to be Ac-7, since it comigrated with mature F pilin. Small amounts of JEL93-reactive Ac-7 pilin have also been detected in inner membranes prepared from cells carrying other TraA$^+$ TraX$^+$ clones (17). TraX also increased the amount of 13-kDa product remaining intact for more than 5 min after labeling, although these polypeptides seemed to be in a configuration or location inappropriate to signal sequence processing.

The stability of the two 7-kDa products [7(Q) and Ac-7] which persist in TraA$^+$Q$^+$ and TraA$^+$X$^+$ cells resembled that of the pool of Ac-7 F pilin observed in TraA$^+$Q$^+$X$^+$ cells. The inner membrane F-pilin subunits of F*lac* cells are, similarly, very stable (25). Thus, once processed correctly, membrane pilin polypeptides may be intrinsically stable; we expect that such subunits might be stacked together and protected by association with each other as well as their membrane environment. However, our data do not preclude the possibility that interactions with other *tra* proteins normally contribute to subunit stability.

Two extremely interesting conclusions that can be drawn from our experiments are first, that the 8(Q) and Ac-8 pilin polypeptides result from an additional modification(s) of a 7-kDa pilin polypeptide, and second, that 7(Q) is the best (and possibly the only) substrate for such modification. Thus, in TraX$^+$ cells, the amount of Ac-8 could depend on how effectively TraX competes with the modifying enzyme and manages to acetylate 7(Q) prior to its modification. Since acetylation would increase the hydrophobicity of the pilin N terminus, it might interfere with additions to pilin by altering the exposure of requisite residues. Although the nature of the modification is not known, our data did suggest that multiple additions might occur at one or several sites on the polypeptide. A host protein(s), not *tra* proteins, would appear to be involved, since modification occurred in cells carrying only the *traA* and *traQ* genes of F. It has long been suggested that purified pilus preparations contain some phosphate and glucose as well as tightly associated lipopolysaccharide (2, 3, 5), although more recent electron spectroscopic imaging results seem to rule out phosphate modification (6). Since our data indicate that only a subset of subunits is altered, it will be interesting to test 8-kDa subunits for these and other types of modification; flagella, for instance, contain the unusual amino-acid, ε-*N*-methyl-lysine (1).

In any case, it is now clear that pili normally contain both modified and unmodified subunits. Pili purified from F*lac* strains have been shown to contain a minor number of Ac-8 subunits in addition to Ac-7 proteins (17), whereas preparations from a TraX$^-$ strain seem to contain a more equivalent quantity of 8(Q) and 7(Q) subunits (18). The slightly different structural properties reported for pili expressed from a clone lacking *traX* and other distal *tra* genes (9) could therefore reflect an increase in the number of modified subunits, as well the absence of the N-terminal acetyl group.

The purpose of having two different kinds of F pilin in a variable ratio still remains unclear. It may be that the two subunit types increase the flexibility of F pili formed in response to the environment. Studies of microtubule assembly in *Saccharomyces cerevisiae* suggest that alpha tubulin may have evolved from beta tubulin by gene duplication to refine the properties of a preexisting microtubule that was composed solely of beta tubulin subunits. This may allow more linear, rigid tubules to form, generate polarity, or facilitate interactions with factors modulating microtubule assembly (26). Conditions affecting the ratio of 7- to 8-kDa pilin subunits remain to be explored. It will be interesting to test whether alternative growth conditions change this ratio and whether the 8-kDa proteins contribute structural or adhesive properties to pili that affect conjugation.

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