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Role of the Propilin Leader Peptide in the Maturation of F Pilin

NADIM MAJDALANI,* DEANNA MOORE, 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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F-pilin maturation and translocation result in the cleavage of a 51-amino-acid leader sequence from propilin and require LepB and TraQ but not the SecA-SecY secretion pathway. The unusual propilin leader peptide and the dependence of its cleavage on TraQ suggested that TraQ recognition may be specific for the leader peptide. An in vitro propilin cleavage assay yielded propilin (13 kDa), the pilin polypeptide (7 kDa), and a 5.5-kDa protein as the *traA* products. The 5.5-kDa protein comigrates with the full-length 51-amino-acid leader peptide, and [^{14}C]proline labeling confirmed its identity since the only proline residues of propilin are found within the leader peptide. The in vitro and in vivo propilin-processing reactions proceed similarly in a single polypeptide cleavage step. Furthermore, TraQ dependence is a property of F-pilin maturation specifically rather than a property of the leader peptide. A propilin derivative with an amino-terminal signal sequence generated by deleting codons 2 to 28 required TraQ for processing in vivo. On the other hand, a chimeric protein with the propilin wild-type leader peptide fused to the mature portion of β-lactamase was processed in a TraQ-independent manner. Thus, despite its unusual length, the propilin leader peptide seems to perform a function similar to that of the typical amino-terminal signal sequence. This work suggests that TraQ is not necessary for the proteolysis of propilin and therefore is likely to act as a chaperone-like protein that promotes the translocation of propilin.

Bacterial conjugation, mediated by the F plasmid, requires the elaboration of surface pili. These F pili initiate the contact between donor and recipient cells that ultimately leads to F DNA transfer. In the absence of pili, cells are transfer deficient. The F pilus is assembled from F-pilin subunits that are present as pools in the cytoplasmic membrane (21, 22). The subunits are derived from the *traA* gene product (4), a 121-amino-acid (aa) polypeptide that is processed to a 7-kDa peptide (51 aa) in the presence of the F-encoded TraQ protein (11, 15, 29, 30). This pilin peptide is further modified by acetylation by TraX to yield the mature F-pilin subunit (14, 20). In the preceding paper (12), propilin processing was shown to be dependent on leader peptidase (LepB) as well as on an established proton motive force. Some data in the preceding paper also indicated that processing occurred as a single polypeptide cleavage event, suggesting no role for TraQ-dependent proteolytic activity on the propilin leader peptide.

Although the Ala-52 residue was identified by peptide sequencing to be the first residue of F pilin (4), and although we had shown in the study described in the preceding paper that LepB is involved in the cleavage of the propilin peptide (12), little was known about the fate of this peptide. In particular, leader peptide processing could occur in two steps: TraQ could initiate a first cleavage within the leader peptide, and LepB would then cleave the exposed signal sequence. To examine this possibility, we developed an in vitro assay and refined our in vivo assay to better detect the leader peptide. We also characterized the TraQ dependence of altered propilins that lacked portions of their leader peptide and constructed various protein fusions to determine the function of the leader peptide.

MATERIALS AND METHODS

Bacterial strains and plasmids. The strains used in this study are listed in Table 1. Plasmid constructions are summarized in Table 2. Plasmids pUC4K and pUC4KIXX were purchased from Pharmacia Molecular Biology Division (Piscataway, N.J.). Plasmids pSport1 and pBluescript II SK$^+$ were purchased from Gibco/BRL Life Technologies (Gaithersburg, Md.) and Stratagene Cloning Systems (La Jolla, Calif.), respectively. Plasmid pYZ5 was generously provided by Jenny Broome-Smith (31).

Media. LB broth and JMM minimal medium were as described previously (●).

Mutagenesis. Oligonucleotide-directed mutagenesis was done as described by Kunkel et al. (9, 10). The primers were AS1 (5′-CCGAAATTGAGGTAACT TATGCTTCGCCTGGCTCGCGCAGTG-3′) for generating pSM141 and AS2 (5′-CCGAAATTGAGGTAACTTATGGCTCGCG-CAGTGATCCCGGATG-3′) for generating pSM142.

DNA sequence analysis. DNA sequencing was performed by the Gene Technologies Laboratories, Texas A&M University (College Station). Deletion mutations in plasmids pSM141 and pSM142 were sequenced by using the T7 promoter primer (5′-TAA TAC GAC TCA CTA TAG GG-3′C); amber linker insertions in pKI504 and pKI511 were sequenced by using the pilin1 primer (5′-GGA GTC CTT ACC GAA GGT CGC-3′).

In vivo labeling of Tra-Bla fusions and *tra*-encoded polypeptides. Protein labeling in strain XK100 was as described previously (13) but with slight modifications: IPTG (isopropyl-β-D-thiogalactopyranoside) induction for 20 min and rifampin treatment for 40 min were performed prior to the 1-min labeling period; whole-cell fractions were suspended in 200 μl of sample buffer, and 5 μl per lane was loaded on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels for analysis. The cells poorly tolerated pKI520, which occasionally led to poor labeling.

Procedures for in vitro labeling of gene products. Plasmid products were labeled with [^{35}S]methionine in vitro by using a prokaryotic-DNA-directed translation kit purchased from Amersham Life Sciences (Arlington heights, Ill.) according to the manufacturer's specifications. For [^{14}C]proline labeling, a proline-deficient amino acid mixture containing all amino acids at 5 mM but lacking proline was made. This mixture replaced the one provided by Amersham. Reaction mixtures containing 1% Triton X-100 (32) were prepared by adding 5 μl of a 6% Triton X-100 stock solution (in Amersham's dilution buffer) to substitute for a similar volume of dilution buffer in a standard 30-μl reaction mixtures. A 5-μl aliquot of the reaction mixture was mixed with 20 ml of acetone, chilled for 15 min, and centrifuged at 4°C for 10 min. Pellets were air dried and suspended in 200 ml of sample buffer.

* Corresponding author. Present address: Laboratory of Molecular Biology, National Cancer Institute, Bldg. 37, Room 2D26, 37 Convent Dr., Bethesda, MD 20892-4255. Phone: (301) 496-4984. Fax: (301) 496-2212. Electronic mail address: nadim@helix.nih.gov.

† Present address: Department of Biotechnology, Burapha University, Bangsaen, Chonburi 20131. Thailand.

‡ Deceased.

TABLE 1. Bacterial strains

Strain	Genotype	Reference
ED2149	F$^-$ *lac*Δ*U124* [*nadA aroG gal att*λ *bio*]	28
JC3051	F$^-$ *his trp* Δ*lac*-X74 *rpsL tsx* λ^r *mal*	1
TB1	*ara* Δ(*lac-proAB*) *rpsL lacZ* M15 (r_K^- m_K^+)	8
XK100	Spectinomycin-resistant *E. coli* B derivative of BL21(DE3) host that carries a T7 RNA polymerase gene in its chromosome	16
XK1200	Spontaneous Nal^r derivative of ED2149	7

Preparation of subcellular protein fractions. Periplasmic proteins from labeled cell cultures were isolated by osmotic shock (16).

Bacterial mating and complementation experiments. Quantitative matings were as previously described (7). Strains ED2149 and JC3272 were used as donor strains; JC3051 was the recipient strain. Plasmids were introduced by calcium chloride transformation (17). IPTG (final concentration, 1 mM) was added 2 h prior to mating to induce P_{Lac} expression of the *traA* genes cloned on the pSport1 (pSM88, pSM141, and pSM142) or the pBluescriptII KS$^+$ (pKI513, pKI514, and pKI515) vector.

Construction of a leaderless propilin derivative. We constructed plasmid pKI511 by introducing a linker at the unique *Nae*I site in *traA* on plasmid pKI503N (Table 2). The linker sequence encodes a TGA translation stop codon at its 5′ end, a consensus Shine-Dalgarno sequence, an *Nco*I recognition sequence for screening, an ATG translation start codon, and the GCC codon of the Ala-52 residue (removed by the *Nae*I digestion) at the 3′ end. This linker was termed *traA* leader stop 4, or AS4. However, this construct is under the control of the temperature-inducible promoter, and, for consistency, we preferred to use IPTG-inducible promoters. Thus, we proceeded to construct derivatives of pKI511. An *Xho*I-*Bam*HI DNA fragment from plasmid pKI511 was cloned into the *Xho*I-*Bam*HI sites distal to the P_{Lac} promoter on the pBluescriptII KS$^+$ vector to yield plasmid pKI513. With this plasmid, gene expression was now IPTG inducible. A deletion extending from the *Afl*II site in the *traL* sequence to the *Bam*HI site distal to the *traQ* sequence on plasmid pKI513 was made to generate plasmid pKI514. This clone does not express *traQ*. Plasmid pKI515 carried a larger internal deletion extending from the *Nco*I site within the AD4 linker to the *Bam*HI site distal to *traQ* on pKI513; it can express only the leader sequence.

RESULTS

TraQ-independent processing in vitro generates a 5.5-kDa leader peptide and the 7-kDa pilin polypeptide. To test whether the propilin polypeptide can be processed in the absence of TraQ, we used plasmid pKI507 (*traYAL*$^+$) or pKI503N (*traYAL*$^+$ *traQ*$^+$) as the template for *traA* expression in a coupled in vitro transcription-translation system. Under standard conditions, the pattern of TraA production from

TABLE 2. Plasmids

Plasmid	Description	Reference
pKI002	TraY$^+$A$^+$L$^+$Q$^+$; *Xho*I-*Bam*HI 2.8-kb *traYAL* + *traQ* fragment from pKI503N in pBluescriptII SK$^+$ *Xho*I-*Bam*HI; *traA* and *traQ* expressed from the T7 promoter	This study
pKI158	TraQ$^+$; pACYC-derivative, constitutively expresses *traQ*	12
pKI500	Expression vector; carries a λ p_L promoter and the λ *c*I857 repressor gene	12
pKI501	T7 RNA polymerase positive; pACYC177-based plasmid carries a T7 RNA polymerase gene controlled by the *lacUV5* promoter and a copy of the *lacI*^{q} repressor gene	13
pKI503N	TraY$^+$A$^+$L$^+$Q$^+$; derivative of pKI500, expresses genes from the λ p_L promoter	12
pKI504	Leader peptide positive, TraA$^-$; *Xba*I [amber codon linker 1062 d(CTAGTCTAGACTAG) (New England Biolabs)] linker insertion at *Nae*I site in pKI503N *traA*; expresses the leader peptide from the λ p_L promoter	This study
pKI507	TraY$^+$A$^+$L$^+$; derivative of pKI500, expresses *traYAL* from the λ p_L promoter	12
pKI511	Leader peptide positive, 7-kDa peptide positive; *Nco*I linker sequence d (**TGA**GGAGGTATTCC**ATG**GCC) at the *Nae*I site in pKI503N; TGA codon termination of leader peptide, leaderless pilin peptide restarts at the ATG codon	This study
pKI513	Leader peptide positive, 7-kDa peptide positive, TraL$^+$ TraE$^+$ TraQ$^+$; *Xho*I-*Bam*HI 3.2-kb fragment from pKI511 (*traA-Nco*I linker, *traLE* + *traQ*) into *Xho*I-*Bam*HI of pBluescriptII KS$^+$ under P_{Lac} control (Fig. 8A)	This study
pKI514	Same as pKI513 but TraQ$^-$; *Afl*II-*Bam*HI pKI513 end filled, religated; removes *traE*, *traL*, and *traQ* (Fig. 8A)	This study
pKI515	Leader peptide positive; *Nco*I-*Bam*HI pKI513 end filled, religated; expresses only the propilin leader peptide (Fig. 8A)	This study
pKI517	*Sma*I 0.9-kb Cm^r cassette from pUC4CIXX into pSM141 *Bsp*HI; Cm^r replaces Ap^r of pSport1 (Gibco/BRL)	This study
pKI518	*Sma*I 0.9-kb Cm^r cassette from pUC4CIXX into pSM142 *Bsp*HI; Cm^r replaces Ap^r of pSport1 (Gibco/BRL)	This study
pKI519	Altered leader peptide 141-BlaM$^+$; *Pvu*II-*Sma*I 0.9-kb β-lactamase from pYZ5 into pKI517 *Nae*I; mature *blaM* fused to the altered leader from pSM141 (codons 2–28) downstream from the T7 promoter	This study
pKI520	Altered leader peptide 142-BlaM$^+$; *Pvu*II-*Sma*I 0.9-kb β-lactamase from pYZ5 into pKI518 *Nae*I; mature *blaM* fused to the altered leader from pSM142 (codons 2–31) downstream from the T7 promoter	This study
pSM88	TraA$^+$L$^+$E$^+$; *Pst*I 1.45-kb *traALE* fragment from pKI268 in pSport1 *Pst*I site; *traALE* expression from the *lac* and T7 promoters	This study
pSM104	Propilin leader peptide-BlaM$^+$; *Pvu*II-*Sma*I 0.9-kb β-lactamase fragment from pYZ5 in pSM103 *Nae*I site; *traA* signal fused to *bla* expressed from the *lac* and T7 promoters	This study
pSM141	Altered propilin leader peptide 141; Oligonucleotide-directed deletion of codons 2–28 of the propilin leader from pSM88	This study
pSM142	Altered propilin leader peptide 142; Oligonucleotide-directed deletion of codons 2–31 of the propilin leader from pSM88	This study
pUC4CIXX	Derivative of pUC4KIXX carrying an *Nsp*V 0.9-kb Cm^r fragment from pBR325 (Gibco/BRL) used to replace the *kan* cassette on pUC4KIXX; source for Cm^r	15
pYZ5	Plasmid carrying promoterless mature *blaM* as a cassette; from J. K. Broome-Smith	31

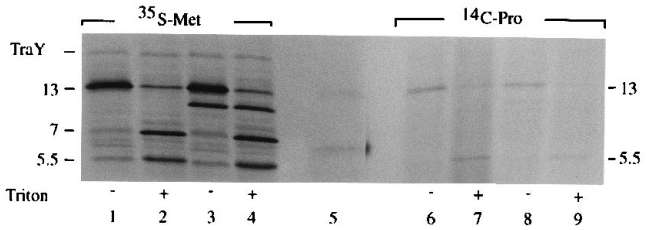

FIG. 1. In vitro processing of propilin. The TraA products were labeled with [^{35}S]methionine or [^{14}C]proline as indicated. The products of plasmid pKI507 (*traA*$^+$) in the absence (−) or the presence (+) of Triton X-100 are shown in lanes 1, 2, 6, and 7. Lanes 3, 4, 8, and 9 show the products of plasmid pKI503N (*traA*$^+$ *traQ*$^+$) under similar conditions. The ^{14}C-methylated cytochrome *c* (12.5 kDa) and aprotinin (6.5 kDa) (Amersham) are shown in lane 5. Positions of the 13-, 7-, and 5.5-kDa TraA products are indicated. TraQ is the unmarked band of about 12.5 kDa visible in lanes 3 and 4. The right half of the gel was exposed for 3 months with the left side covered. A day before developing, the left side was then exposed.

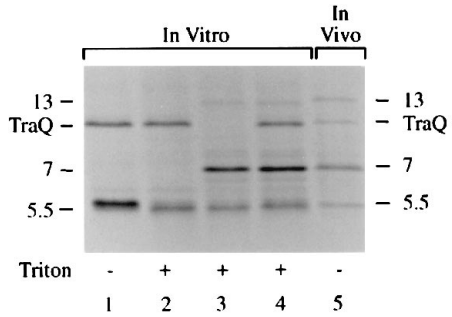

FIG. 2. In vivo processing of propilin. The autoradiograph compares TraA products synthesized in vitro and in vivo. Plasmid pKI504 encodes a polypeptide corresponding to the 54-aa uncleaved leader peptide (lane 1) or a 51-aa peptide cleaved in the presence of Triton X-100 (+) (lane 2). Products of plasmids pKI507 (lane 3) and pKI503N (lane 4) are the same as shown in Fig. 1 (lanes 2 and 4). Lane 5 shows polypeptides encoded by plasmid pKI002 and labeled in vivo in strain XK100 in the presence of rifampin. Numbers at the sides indicate sizes (in kilodaltons).

pKI507 was essentially the same as that observed when *traA* is expressed in TraQ$^-$ cells in vivo: the 13-kDa polypeptide was the primary product, and only a small fraction of this was processed to the 7-kDa pilin polypeptide (Fig. 1, lane 1). Concurrent synthesis of the TraQ protein had no effect on the relative amounts of the 13- and 7-kDa polypeptides under these conditions (Fig. 1, lane 3), although the 11-kDa TraQ protein was also synthesized from the pKI503N template (Fig. 1, lane 3). Synthesis of TraY, which is encoded by both plasmids, was also detected, but, as in in vivo experiments, no TraL was detected.

Since propilin is a very hydrophobic peptide and is likely to be insoluble in aqueous buffers, we repeated these experiments in the presence of the nonionic detergent Triton X-100. Addition of this solubilizing agent had a profound effect on the *traA*-derived polypeptides synthesized from both the pKI507 and pKI503N plasmids (Fig. 1, lanes 2 and 4). With either template, much less labeled propilin (13 kDa) was observed, and the intensities of the pilin band (7 kDa) and of a band migrating at approximately 5.5 kDa increased significantly.

The 5.5-kDa peptide is the propilin leader peptide. The 5.5-kDa polypeptide matched the predicted size of the full-length propilin leader peptide. To confirm that the 5.5-kDa band represents the 51-aa propilin leader peptide, [^{14}S]proline labeling, which occurs only in the leader, was used to distinguish pilin from the leader peptide. Lanes 6 to 9 of Fig. 1 show the [^{14}C]proline-labeled products synthesized in vitro from the pKI507 and pKI503N plasmids. In the absence of Triton X-100, only propilin (13 kDa) was detected (lanes 6 and 8). In the presence of detergent, less labeled propilin accumulated, and labeled 5.5-kDa peptide was easily visible (lanes 7 and 8). As expected, the 7-kDa pilin polypeptide was not labeled. This experiment is the first unambiguous demonstration that the entire propilin leader peptide is removed from propilin in a single proteolytic cleavage step.

Since no genotype is available for the MRE600 strain used in preparing the in vitro transcription-translation extract, we tested and confirmed that it was phenotypically F$^-$ and carried no TraQ-like activity in vivo (data not shown). Thus, it is clear from these experiments that a detergent-activated protease is present in the *Escherichia coli* extracts used for in vitro synthesis and is capable of processing propilin correctly. This enzyme cannot be TraQ; the specificity of the cleavage and the conditions under which the protease becomes active both suggest that it is LepB (25, 32). Cell-free S-30 extracts are not totally membrane free and thus may contain some LepB associated with these membrane fractions.

TraQ-dependent maturation also generates a 5.5-kDa leader peptide in vivo. To provide an independent measure of the size of the leader peptide, we inserted an amber codon linker into plasmid pKI503N to generate plasmid pKI504. The linker is three codons distal to the TraA-processing site. Thus, pKI504 encodes a truncated 54-residue *traA* product that includes the normal propilin leader sequence and signal-processing site. Figure 2, lane 1, shows the predicted 54-residue *traA* product synthesized in vitro in the absence of Triton X-100. It migrated slightly more slowly than the same product synthesized in reaction mixtures containing Triton X-100 (Fig. 2, lane 2), suggesting that the 54-residue peptide is probably cleaved down to 51 aa. This latter product comigrated exactly with the 5.5-kDa polypeptide produced by the *traA* gene under the same Triton X-100 conditions in vitro (Fig. 2, lanes 3 and 4).

Since propilin processing in vitro yielded a 5.5-kDa leader peptide in addition to the expected 7-kDa form of pilin, we refined our in vivo TraA-TraQ expression system to facilitate the detection of the leader peptide. In these experiments, the *traA* and *traQ* genes were expressed from the T7 promoter of pKI002. This procedure allowed us to label TraA-derived peptides in the presence of rifampin, which reduces the background of labeled host proteins that may obscure detection of the leader peptide. Figure 2, lane 5, demonstrates that expression of *traA* on plasmid pKI002 produces 7- and 5.5-kDa polypeptides, which are the same sizes as those seen in TraQ-independent processing in vitro (Fig. 2, lanes 2 and 3).

Therefore, propilin processing in vivo does proceed as a single polypeptide cleavage event, clearly indicating that there is no requirement for a second proteolytic event that would be mediated by TraQ. Furthermore, our results suggest that the 54-aa peptide contains all of the information necessary for cleavage.

Construction and functional analysis of propilin leader sequence deletion mutants. The 51-aa leader peptide of propilin contains what appears to be a conventional signal sequence from Arg-30 to Ala-51. However, unlike that of other exported proteins, the putative signal sequence of propilin begins some 28 residues distal to the initiator methionine. This unusual positioning of the signal sequence might account for the TraQ-dependent, Sec-independent insertion of propilin into the cytoplasmic membrane. To test whether the unusual N-terminal

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pSM88    MNAVLSVQGASAPVKKKSFFSKFTRLNMLRLARAVIPAAVLMMFFPQLAMA51 AGSSGQ
pSM141   M--------------------------LRLARAVIPAAVLMMFFPQLAMA51 AGSSGQ
pSM142   M----------------------------ARAVIPAAVLMMFFPQLAMA51 AGSSGQ
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FIG. 3. Amino acid sequences of the wild-type propilin leader peptide (pSM88) and two deletion mutants (pSM141 and pSM142). The cleavage site is located at Ala-51 as determined previously (4). Dashed lines represent the deleted codons.

sequence of propilin was required for TraQ recognition, we used oligonucleotide-directed mutagenesis to introduce deletion mutations in plasmid pSM88 (*traA*$^+$). To avoid introducing changes that might affect the expression of the altered propilins, the transcriptional and translational initiation sites for *traA* were not altered. However, the deletions modified the length and appearance of the propilin leader so that it resembled a more conventional signal sequence. One derivative, in plasmid pSM141, lacked *traA* codons 2 to 28 (*traA Δ2-28*) another, in plasmid pSM142, lacked *traA* codons 2 to 31 (*traA Δ2-31*) (Fig. 3).

The ability of the altered propilins to substitute for wild-type propilin was assessed with strains carrying F*lac traA1* or F*lac traQ238* (Table 3). Under the conditions tested, plasmid pSM141 (*traA Δ2-28*) complemented F*lac traA1* almost as well as the parental pSM88 plasmid (*traA*$^+$). Thus, the modified propilin encoded by plasmid pSM141 appeared to be an acceptable precursor for pilin formation in this host. In previous work, it was shown that overexpression of *traA* could partially overcome a TraQ mutation (19). We confirmed this with plasmid pSM88 (*traA*$^+$), which partially restored conjugation to an F*lac traQ238* host. However, overexpression of the *traA Δ2-28* product from pSM141, in this background, failed to restore conjugative DNA transfer (Table 3). Apparently, in TraQ$^-$ cells, more F pilin can be derived from the wild-type propilin expressed from plasmid pSM88 (*traA*$^+$) than from the altered propilin expressed from pSM141 (*traA Δ2-28*). The *traA Δ2-31* product expressed from plasmid pSM142 did complement F DNA transfer from either F*lac traA1* or F*lac traQ238* cells to above the 1% level that we consider significant. This result suggests that TraA Δ2-28 is still TraQ dependent.

Processing of altered propilins. We next examined the ability of the TraA Δ-28 and TraA Δ2-31 polypeptides to be processed to the 7-kDa form of pilin in vitro and in vivo.

Synthesis of TraA Δ2-28 and TraA Δ2-31 in vitro yielded uncleaved 8.5- and 8.0-kDa polypeptides, respectively, in the absence of detergent. The addition of Triton X-100 resulted in the accumulation of the 7-kDa pilin polypeptide from both products (data not shown). Thus, both TraA Δ2-28 and TraA Δ2-31 were processed in vitro, suggesting that the deletions had no deleterious effects on the recognition and use of the processing site.

TABLE 3. Altered propilin complementation of F*lac traA1* and F*lac traQ238*

Donor plasmid[a]	Transfer (% donors)[b]
F*lac*	115
F*lac traA1*	$<2 \times 10^{-5}$
F*lac traA1* + pSM88	21.0
F*lac traA1* + pSM141	7.2
F*lac traA1* + pSM142	0.01
F*lac traQ238*	0.004
F*lac traQ238* + pSM88	5.0
F*lac traQ238* + pSM141	0.001
F*lac traQ238* + pSM142	0.003

[a] The donor strain was XK1200 Nal^r Lac$^+$ (Amp^r when transformed with any of the plasmids listed).

[b] The recipient strain JC3051 is Strp^r Lac$^-$. Matings were performed as described in Materials and Methods. Transconjugants were selected as Strp^r Lac$^+$ colonies and were patched to check that they were Amp^s.

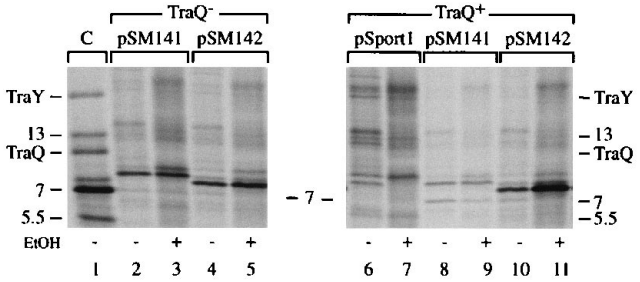


FIG. 4. The TraA Δ2-28 and TraA Δ2-31 propilin products expressed in vivo in TraQ$^-$ (XK100) and TraQ$^+$ (XK100/pKI158) cells. The products expressed from plasmids pSport1 (vector [lanes 6 and 7]), pSM141 (TraA Δ2-28 TraQ$^-$ [lanes 2 and 3 and TraQ$^+$ [lanes 8 and 9]) and pSM142 (TraA Δ2-31 TraQ$^-$ [lanes 4 and 5] and TraQ$^+$ [lanes 10 and 11]) are shown. Synthesis in the absence (−) or presence (+) of ethanol (ETOH) is indicated. Numbers at the sides indicate sizes (in kilodaltons).

When the modified *traA* genes of plasmids pSM141 (*traA Δ2-28*) and pSM142 (*traA Δ2-31*) were expressed in a TraQ$^-$ host in vivo, the same pilin-related bands at approximately 8.5 and 8 kDa were seen in vitro; only very faint bands at 7 kDa were detected (Fig. 4, lanes 2 to 5). On the other hand, in a TraQ$^+$ host, a significant fraction of TraA Δ2-28 was processed to 7-kDa pilin (Fig. 4, lane 8), and this processing was not abolished by ethanol (lane 9). In contrast, TraQ had no discernable effect on the 8-kDa product expressed from plasmid pSM142 (TraA Δ2-31). As described below, this signal sequence could not function in the export of β-lactamase, indicating a general defect in it. Thus, the ability of the altered propilins to complement F*lac traA*1 or F*lac traQ238* was correlated with the production of the 7-kDa form of pilin in vivo.

The stabilities of the altered propilins were determined in a pulse-chase experiment with a TraQ$^-$ host. As with wild-type propilin (12), degradation of the altered propilins was rapid, with no detectable increase of the 7-kDa polypeptide during the chase period (data not shown).

Processing of the *traA*::*bla* fusion proteins. To test the effect of the propilin leader sequence and its deletion derivatives on the secretion and processing of a *sec*-dependent secretory protein, a series of *traA*::*bla* gene fusions was constructed. Plasmid pSM104 encodes a protein with a wild-type propilin leader sequence and processing site (residues 1 to 52) preceding the sequence of mature β-lactamase. The gene fusions on plasmids pKI519 and pKI520 fuse the *traA Δ2-28* and the *traA Δ2-31* leader sequences, respectively, proximal to the sequence of mature β-lactamase.

As in the case of the wild-type *bla* precursor (Fig. 5, lanes 1 and 2), the 34.5-kDa *traA51*::*bla* fusion product encoded by plasmid pSM104 was processed efficiently in the absence of ethanol, but processing was inhibited in the presence of ethanol (Fig. 5, lanes 3 and 4). Similarly, the 31.9-kDa *traA Δ2-28* product encoded by plasmid pKI519 was also processed in an ethanol-sensitive manner (Fig. 6, lanes 1 and 2). Processing of these fusion products was also tested in the presence of 10 mM sodium azide and was inhibited equally as with ethanol (data not shown). In agreement with the result from pSM142 (*traA Δ2-31*), the 31.5-kDa *traA Δ2-31*::*bla* fusion product encoded by plasmid pKI520 was inefficiently processed (Fig. 6, lanes 3

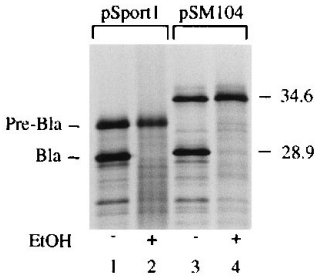


FIG. 5. In vivo processing of the TraA52::Bla fusion protein. Polypeptides expressed from plasmids pSport1 and pSM104 were labeled with [^{35}S]methionine in XK100 cells. Processing was monitored in the absence (−) or presence (+) of ethanol (ETOH). The fusion protein has a molecular mass of 34.6 kDa, while the processed moiety comigrates with the mature β-lactamase at 28.9 kDa.

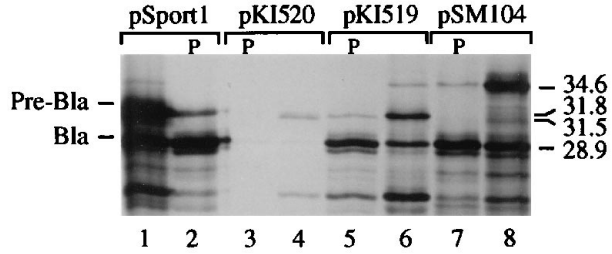


FIG. 7. Subcellular localization of the fusion peptides. Labeled polypeptides encoded by pSport, *traA52*::*bla*, *traA2-28*::*bla*, and *traA2-31*::*bla* were separated by SDS-PAGE. Cells were fractionated by osmotic shock, and samples were separated into a crude pellet fraction and a periplasmic fraction (P). Positions predicted for the various precursors of fusion proteins are indicated by size markers (in kilodaltons).

and 4). Furthermore, cells carrying plasmids pSM104 and pKI519 were Ap^r on selective plates, indicating that some of the fusion product was secreted and functional normally in the absence of TraQ. In contrast, cells carrying plasmid pKI520 required induction from the P_{Lac} promoter upstream of the fusion gene in order to develop the Ap^r phenotype, indicating that this product is not easily processed and/or exported to the periplasm (data not shown).

To confirm the periplasmic localization of the processed *traA*::*bla* fusion products, we isolated labeled periplasmic proteins from cells expressing the various gene fusions. The results of the osmotic shock fractionation are shown in Fig. 7. The different precursors were enriched in the cell pellet fraction (lanes 1, 4, 6, and 8), whereas processed fusion peptides were enriched in periplasmic fractions (lanes 2, 5, and 7). This result indicated that the wild-type propilin leader and an altered leader correctly targeted their respective TraA::Bla fusion proteins for export.

These results indicate that the propilin leader peptide can replace the β-lactamase signal sequence with no requirement for TraQ. Also, processing of the fusion protein is Sec dependent. Therefore, the TraQ dependence of propilin maturation must be specific to the mature portion of the pilin peptide or to an interaction between the mature portion and the leader peptide.

Construction of a leaderless derivative of propilin. Previously, Derman et al. (3) demonstrated that 1% of an alkaline phosphatase lacking a signal sequence could be exported, possibly via an alternative, non-SecY secretion pathway. Since TraQ seemed to be specific for the mature domain of pilin, it was possible that the leader peptide was dispensable. Thus, we examined the translocation ability of a leaderless pilin by measuring the ability of such a peptide to complement either an F*lac traA1* or an F*lac traQ238* mutation. For this purpose, we constructed plasmid pKI511, with a linker insertion that terminates the translation of the leader peptide and a Shine-Dalgarno box to allow translation restart into the mature portion of pilin (Fig. 8A). To place this construct under the expression of an IPTG-inducible promoter (see Materials and Methods), we moved the entire fragment to make plasmid pKI513. Internal deletions were then made to remove the *traQ* sequences (pKI514) or to remove the pilin and the *traQ* sequences (pKI515) (Fig. 8A).

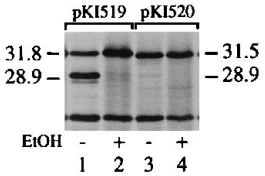


FIG. 6. In vivo processing of the fusion proteins TraA Δ2-28::Bla (encoded by pKI519) and TraA Δ2-31::Bla (encoded by pKI520). XK100 cells carrying the plasmids were used to label the proteins after induction of expression with IPTG. The autoradiograph shows the SDS-PAGE-separated fusion proteins. The predicted sizes of the precursors (in kilodaltons) are indicated. ETOH, ethanol.

We determined the abilities of plasmids pKI513, pKI514, and pKI515 to complement a *traA1* or a *traQ238* mutation (Fig. 8B). No complementation was observed when these constructs were introduced into an F*lac traA1* host strain, indicating that no functional pilin subunits could be derived from a leaderless 7-kDa peptide. Only plasmid pKI513, carrying the *traQ* sequences, can complement the *traQ238* mutation. Therefore, the presence of the amino-terminal leader peptide is necessary to ensure the correct translocation of propilin.

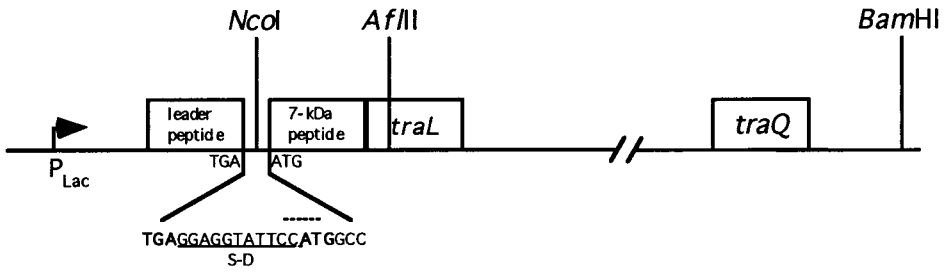
DISCUSSION

The propilin leader peptide is an unusual structure that carries an internal signal sequence. The dependence of propilin processing on the presence of TraQ suggested several possible roles for TraQ. One possibility is that TraQ is an endopeptidase that cleaves within the leader peptide. This initial cleavage could remove the first 25 to 30 amino acids and expose the signal sequence at the amino terminus of the protein. Such a two-step processing reaction is exemplified by the import of cytochrome c_1 and cytochrome b_2 to the intermembrane space in mitochondria (2, 5, 6). A similar two-step processing pathway has also been observed in the export of the **a**-factor in *Saccharomyces cerevisiae* (18).

Our data clearly eliminate the possibility that TraQ is a propilin endopeptidase: the inhibition of leader sequence cleavage leads to the accumulation of a full-length propilin but not of intermediates of smaller size. In the presence of TraQ, cleavage did not increase. Furthermore, our in vitro and in vivo data presented in this paper unequivocally demonstrate that processing occurs in a single polypeptide cleavage step. In vitro, and in the presence of detergent, only two species of [^{14}C]proline-labeled peptides are present: the 13-kDa precursor and the 5.5-kDa leader peptide. Identical peptides are observed in vivo which a T7 expression system. Thus, TraQ is not a propilin endopeptidase.

The unusual length of the leader peptide and the processing dependence on TraQ led us to hypothesize that TraQ dependence was a consequence of the 51-aa leader region of propilin. To test this, we deleted 27 or 30 codons from the amino terminus of the leader peptide to generate propilin derivatives

A



B

Donor	Transfer (% donors)
F *lac traA1*	$<2 \times 10^{-5}$
" + pKI513	0.003*
" + pKI514(Δ*Afl*II-*Bam*HI)	$<2 \times 10^{-4}$
" + pKI515(Δ*Nco*I-*Bam*HI)	$<2 \times 10^{-4}$
F *lac traQ238*	0.004
" + pKI513	2.5
" + pKI514	0.001
" + pKI515	0.001

FIG. 8. (A) Linear map of plasmid pKI513 (not to scale) with the linker sequence. S-D, Shine-Dalgarno consensus sequence; dashed line, *Nco*I restriction site. Other relevant restriction sites are also marked. (B) Transfer efficiencies of plasmid pKI513 and its derivatives in an F*lac traA1* or an F*lac traQ238* mutant. *, no transconjugants, but only recombinants, were obtained.

that carried typical signal sequences. Thus, TraA Δ2-28 and TraA Δ2-31 had at least one positively charged residue preceding a stretch of at least nine hydrophobic amino acids, small, uncharged residues, and the LepB cleavage site (23, 26). These deletions did not affect the LepB processing site as determined in vitro with Triton X-100. However, in vivo, in the absence of TraQ, neither of the altered propilins was conserved to the 7-kDa pilin peptide. With TraQ present, only the TraA Δ2-28 derivative was processed. Furthermore, when the wild-type and the two altered leader peptides were fused to β-lactamase, these chimeras were processed in a SecA-SecY-dependent, TraQ-independent manner. The β-lactamase moiety was correctly translocated to the periplasm and could easily be recovered from osmotic shock fractions. Therefore, our results indicate that TraQ dependence is specific not to the leader peptide per se but rather to the mature pilin domain or to an interaction between pilin and the leader peptide. This suggests that TraQ may have a chaperone-like function.

Purified synthetic peptides cause the opening of an uncharacterized proteinaceous channel in the plasma membrane of *E. coli* (24). Also, an export protein lacking its signal sequence was able to translocate at 1% of wild-type efficiency (3). Combined, these results suggest that signal-sequence-bearing proteins translocate through proteinaceous channels and that signalless proteins can also translocate either through the same channels or via alternate ones. Since TraQ is an inner membrane protein (29), it could well be a propilin-specific channel. Thus, if TraQ interacts with the propilin leader peptide and pilin, then shortening or eliminating the leader would lead to the reduced transfer efficiency we have observed. Alternatively,

TraQ may interact only with pilin, yet a full-length leader would still be required to retard the folding of pilin to maintain it in a translocation-competent form.

Amino-terminal signal sequences have shared characteristics (26) and seem to retard the folding of the mature domains of peptides (3, 27). The 51 aa propilin leader peptide is able to substitute for the signal sequence of β-lactamase and promotes the export of the moiety to the periplasm. Thus, it probably plays the same role in retarding the folding of propilin.

TraQ is not directly involved with propilin leader cleavage yet is necessary for propilin translocation. Thus, it may act to maintain propilin in an insertion-competent form or to form a propilin-specific membrane channel to promote the efficient insertion of propilin.

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