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The Product of the F Plasmid Transfer Operon Gene, *traF*, Is a Periplasmic Protein

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The products of clones carrying the F plasmid transfer operon gene, *traF*, were analyzed. Proteins expressed in maxicells were labeled with [^{35}S]methionine and examined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and autoradiography. Clones carrying the wild-type *traF* gene expressed two polypeptide products that were not products of clones containing the *traF13* amber mutation. These migrated with apparent molecular weights (M_a) of 27,000 and 25,000. A pulse-chase experiment suggested that the larger product was a precursor of the smaller one. In the presence of ethanol, the M_a-27,000 polypeptide accumulated and the M_a-25,000 product was not expressed. These results indicated that the *traF* protein undergoes proteolytic processing associated with export. Cell fractionation experiments further indicated that the greatest concentration of the mature (M_a 25,000) TraF protein was located in the periplasm. The DNA sequence of *traF* and the position of the transition mutation in *traF13* DNA were also determined. Sequence analysis suggested that *traF* would be expressed as a 247-amino-acid, M_r-28,006 polypeptide. The 19 amino acids at the amino terminus of this polypeptide appear to constitute a typical membrane leader peptide, while the remainder of the molecule (M_r 25,942) is predicted to be primarily hydrophilic in character.

Transfer of the conjugative plasmid F requires contact between the donor and recipient cell that is initiated by F-pili extending from the donor cell surface. The expression of at least 14 transfer operon genes, including *traF*, is required for elaboration of these filaments (for review, see references 17 and 45). F *lac traF* mutants are defective in both transfer and F-pilus production and are resistant to RNA and DNA F-pilus-specific bacteriophages (3, 4) but appear to be able to synthesize F-pilin membrane subunits (32).

The *traF* locus has been mapped by analysis of Hfr deletions (16), lambda insertion and transducing phage derivatives (21, 28, 44), and plasmid clones (2, 46). Using a minicell system, Kennedy et al. (22) compared the products of plasmid pRS29 (which includes the large F *Eco*RI fragment, f1) and pRS29 derivatives carrying the amber-suppressible mutations *traF13* and *traF273*. They were able to identify a 25,000-dalton *traF* product associated with minicell envelope fractions. Subsequently, Manning et al. (27) reported minicell membrane fractionation experiments that suggested that TraF was an outer membrane protein.

Early analysis of *tra* operon products suggested that F transfer operon protein export to the outer membrane was not accompanied by signal sequence processing (1). However, subsequent analyses have indicated that these experiments may have been misleading. The *traT* product, a major outer membrane protein involved in surface exclusion, is now known to undergo lipid modification and processing (13, 31, 36). The product of the pilin structural gene, *traA*, is processed when the *traQ* product is also present (18, 24, 33, 46). In contrast, the product of the regulatory gene, *traJ*, appears to be cytoplasmic in normal donor cells; its detection in outer membrane fractions of cells that overproduce the protein may reflect the formation of an insoluble protein complex (9, 10). It was therefore of interest to reexamine the location and product of *traF*, a pilus assembly gene. In this paper we present evidence that the mature *traF* product is a periplasmic protein that is derived from a larger precursor polypeptide. We also report the complete *traF* DNA sequence, which we found encodes a polypeptide with a typical amino-terminal signal sequence.

MATERIALS AND METHODS

Bacterial strains and plasmids. The origin and characteristics of the plasmid derivatives we used are listed in Table 1. The *traF13* derivative of plasmid pRS29 was obtained from N. S. Willetts. The map positions of the *tra* fragments carried by these plasmids are illustrated in Fig. 1. Plasmid vectors used were pBR322 (41) and pACYC177 (7). Strain SE5000 (F$^-$ *Δara-139 ΔlacU169 rpsL relA thi recA56*) and vectors M13mp8 and M13mp9 (30) were obtained from R. Young, Texas A & M University.

Analysis of plasmid products. Plasmid products were labeled with [^{35}S]methionine in maxicells and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and autoradiography as described previously (34, 46). All plasmids were transformed into strain SE5000 for these experiments. The standard labeling period was 60 min unless otherwise indicated. For ethanol experiments, absolute ethanol was added to a 0.2-ml sample of maxicells 1 min prior to addition of label (25 μCi of [^{35}S]methionine per sample), and the samples were incubated for 30 min at 37°C prior to centrifugation. This procedure was modified from the method described by Lory et al. (25).

For pulse labeling, 100 μCi of [^{35}S]methionine was added to a 1.0-ml maxicell culture, and 0.2-ml samples were removed after 2 and 5 min; at 5 min, unlabeled methionine was added to a final concentration of 800 μg/ml, and another set of 0.2-ml samples were removed after an additional 10, 30, and 50 min of incubation. All samples were immediately centrifuged, and the individual cell pellets were frozen at −20°C. Subsequently these were reconstituted in 75 μl of SDS sample buffer, boiled for 3 min, and analyzed by SDS-PAGE (18).

Fractionation of cellular components. Cytoplasmic, periplasmic, and membrane components were separated by a modification of the protocol described by Ito et al. (19).

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TABLE 1. Bacterial plasmids

Plasmid	*tra* fragment size (kb) and ends	Vector and vector junction sites	Comment or reference
pKI165	6.2 *Ava*I [*traF13*(Am)]	pACYC177 *Xma*I	*tra* fragment cloned from pRS29 *traF13* DNA (this paper)
pKI181	2.65 *Pst*I	pACYC177 *Pst*I	46
pKI175	6.2 *Ava*I	pACYC177 *Xma*I	46
pKI184	4.1 *Hin*cII	pACYC177 *Hin*cII	46
pKI185	1.3 *Hin*cII	pACYC177 *Hin*cII	46
pKI272	3.8 *Eco*RV	pBR322 *Eco*RV	26
pKI280	1.5 *Eco*RV, *Sst*I	pBR322 *Eco*RV, *Bam*HI	*Sst*I-*Bam*HI deletion of pKI272 (this paper)
pRS29	15.15 *Eco*RI	pSC101 *Eco*RI	F *Eco*RI f15 and f1 fragments (40)
pRS29 *traF13*	15.15 *Eco*RI	pSC101 *Eco*RI	22

Label (200 μCi of [^{35}S]methionine) was added to 2 ml of maxicells, and the cultures were incubated for 60 min. The cells were then centrifuged at 8,000 rpm for 5 min, washed twice with 0.03 M Tris hydrochloride (pH 8.0), and suspended in 0.2 ml of 20% sucrose buffer (0.03 M Tris hydrochloride, pH 8.0), to which 1/10 volume of 1-mg/ml lysozyme (freshly dissolved in 0.1 M EDTA, pH 7.3) was added. After remaining on ice for 30 min, the suspension was spun in a microcentrifuge for 15 min. The supernatant (periplasmic fraction) was removed and reserved. The pellet was suspended in fresh 20% sucrose buffer and subjected to a second 15-min spin to wash the cells. It was then suspended in 1.2 ml of Tris-EDTA-DTT buffer (10 mM Tris, 5 mM EDTA [pH 7.8], 1 mM dithiothreitol [DTT]), sonicated (32), and centrifuged for 3 min in a microcentrifuge to remove remaining intact cells. This supernatant was centrifuged at 29,000 rpm for 45 min in a Beckman SW55 rotor containing Delrin adaptors for 0.8-ml tubes. The resulting supernatant was reserved for the cytoplasmic fraction. The pellet was washed by suspension in 0.5 ml of Tris-EDTA-DTT buffer, and the SW55 rotor centrifugation step was repeated to collect the membrane pellet fraction.

Prior to use, the periplasmic fraction was diluted with 200 μl of 0.03 M Tris hydrochloride and centrifuged in a Beckman Airfuge (30° A-100 rotor) for 10 min to remove contaminating cells or membrane material. The supernatant periplasmic proteins were then concentrated in a Savant Speed Vac overnight. Similarly, the cytoplasmic fraction was centrifuged for an additional 120 min in the Beckman SW55 rotor (29,000 rpm), and the supernatant fluid containing cytoplasmic proteins was evaporated in the Speed-Vac concentrator. All fractions were suspended in 100 μl of sample buffer and boiled for 3 min immediately prior to SDS-PAGE analysis.

DNA sequencing. M13mp8 and M13mp9 replicative form DNAs were purified by cesium chloride-ethidium bromide ultracentrifugation by the large-scale plasmid preparation procedure described previously (12, 29, 42). Initially, a pKI184 1.3-kilobase (kb) *Eco*RV-*Hin*cII *tra* fragment and a pKI181 1.5-kb *Pst*I-*Sma*I *tra* fragment were cloned on vectors M13mp8 and M13mp9. These were used to obtain *Pst*I, *Taq*I, or *Alu*I fragment subclones. Overlapping segments were sequenced in both directions. DNA sequence determination was done by the dideoxy method of Sanger et al. (38) as modified by Gomer et al. (15). Deoxy- and dideoxynucleoside triphosphates were purchased from Pharmacia, Inc., [α-^{32}P]dATP and [α-^{35}S]dATP were from New England Nuclear Corp., and DNA polymerase I large fragment and

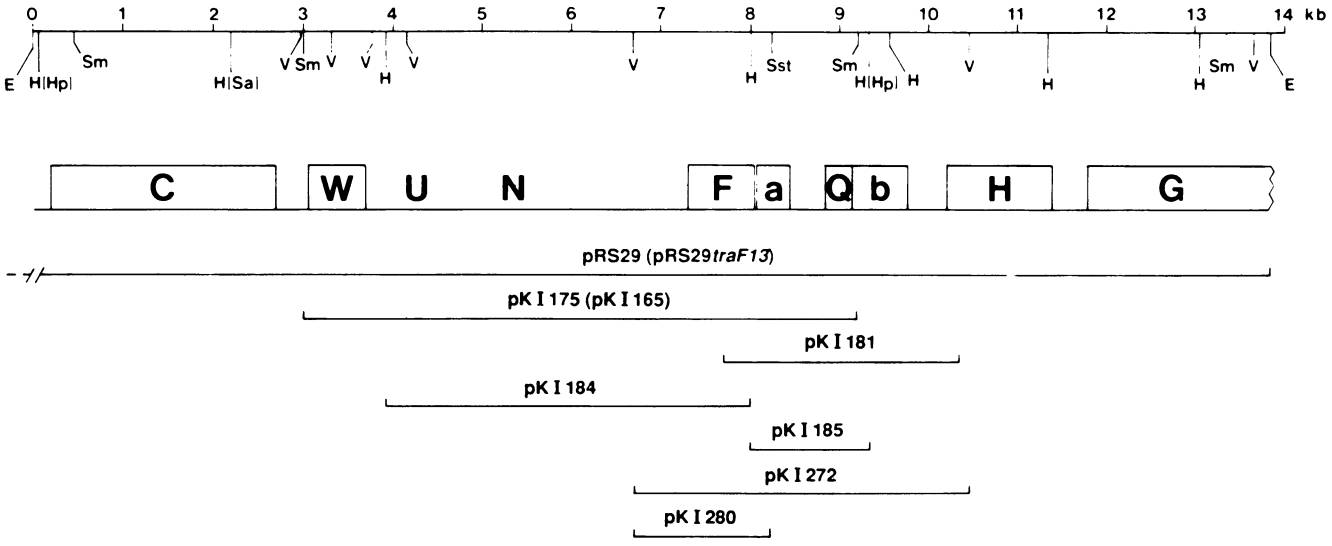

FIG. 1. F *tra* DNA segments carried by plasmids used in this study. Lines in the lower part of the figure indicate the extent of F *tra* DNA carried by each plasmid. The DNA segment of a plasmid named in parentheses carries the *traF13* mutation. The line at the top indicates kilobase coordinates for F *Eco*RI fragment f1 and the positions of restriction sites for *Eco*RI (E), *Eco*RV (V), *Hin*cII (H), *Hpa*I (Hp), *Sal*I (Sa), *Sma*I (Sm), and *Sst*I (Sst). Capital letters indicate *tra* genes *C*, *W*, *U*, *N*, *F*, *Q*, *H*, and *G*; a and b indicate *trb* genes identified by Wu et al. (46).

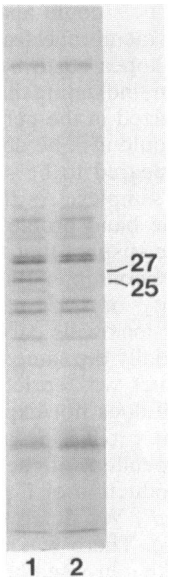

FIG. 2. Autoradiogram of pKI175 and pKI165 products labeled in maxicells and fractionated by SDS-PAGE. Cells carrying plasmid pKI175 (lane 1) express a 27-kDa and a 25-kDa polypeptide. These are not present in samples from cells carrying the *traF13* amber derivative, pKI165 (lane 2).

M13 15-base-pair (bp) primer were from Bethesda Research Laboratories. The sequencing gel electrophoresis system model S1 (20 by 80 cm; Bethesda) and buffer gradient gel (6) were used.

RESULTS

Identification of a TraF precursor. We previously reported the construction of a series of plasmid derivatives that carry various F *tra* operon DNA restriction fragments derived from the large F *Eco*RI fragment, f1 (Fig. 1). One such plasmid, pKI175, carries a 6.2-kb *Ava*I *tra* fragment which includes genes *traW* through *traQ* (26, 46). Analysis of the [^{35}S]methionine-labeled proteins synthesized in maxicells carrying pKI175 has shown that the plasmid expresses a number of *tra*-specific polypeptides; among these, proteins with an apparent molecular weight (M_a) of 23,000, 14,200, and 12,500 have been specifically identified as the products of *traW*, *trbA*, and *traQ*, respectively (26, 46). We also tentatively identified an M_a-25,000 pKI175 product as TraF, since it corresponded to the size that Kennedy et al. (22) associated with the *traF* gene product, and complementation and recombination analysis indicated that expression of the protein was associated with *traF* (46).

In order to confirm the *traF* product identification, we constructed pKI165, a plasmid identical to pKI175 except that the *tra* DNA fragment in pKI165 was derived from the pRS29 *traF13* mutant constructed by Kennedy et al. (22). As shown in Fig. 2, comparison of the polypeptides labeled in maxicells carrying pKI175 or pKI165 demonstrated that pKI165 did not express the M_a-25,000 polypeptide band expressed by pKI175. However, an M_a-27,000 polypeptide band was also missing from pKI165 samples; otherwise the polypeptide pattern was identical to that obtained with pKI175. This result suggested to us that the product of *traF* might first be expressed as a 27-kilodalton (kDa) precursor protein and processed to the 25-kDa form.

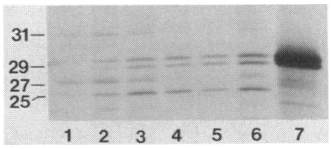

FIG. 3. The 27-kDa product is a precursor of the 25-kDa product. Maxicells carrying pKI175 were labeled with [^{35}S]methionine for 2 min (lane 1) or 5 min (lane 2). After 5 min to incorporate label, cold methionine was added, and additional samples were removed after 10 min (lane 3), 30 min (lane 4), or 50 min (lane 5). Control cultures, labeled for 60 min, carried pKI175 (lane 6) or the vector pACYC177 (lane 7). The positions of the 27-kDa and 25-kDa *traF* products and of the β-lactamase precursor (31 kDa) and mature doublet bands (29 kDa) are indicated.

We tested this hypothesis in a pulse-label experiment. As shown in Fig. 3, the 27-kDa pKI175 product band was prominent and the 25-kDa product was barely detectable in a sample collected after a 2-min labeling period (lane 1). Both bands were readily observed in a sample labeled for 5 min (lane 2). We also added an excess of unlabeled methionine to a culture after a 5-min period of [^{35}S]methionine incorporation and removed samples after a further 10-min (lane 3), 30-min (lane 4), or 50-min (lane 5) chase period. In these samples, the 27-kDa band appeared to decrease in intensity as the chase period progressed, while the intensity of the 25-kDa band initially increased slightly and then appeared to remain relatively constant. As pKI175 also expresses the vector *bla* gene, changes in the intensity of *bla* product bands could be followed in the same samples. The intensity of the β-lactamase precursor band (31 kDa) decreased in parallel with the intensity of the 27-kDa polypeptide. Similarly, the intensity changes in the 25-kDa band were mimicked by the observed intensity of the doublet bands representing the two conformations of processed (29-kDa) β-lactamase (37) that were resolved on our gels. The periplasmic β-lactamase enzyme is known to be derived from the 31-kDa *bla* product through removal of a 23-amino-acid signal peptide (5, 41). Therefore, our pulse-chase experiment strongly suggested that the difference between the 27-kDa and 25-kDa *traF* product bands resulted from a similar leader peptidase processing event.

This interpretation was substantiated by experiments with ethanol. Ethanol perturbs the membrane and impairs secretion and precursor protein processing of various *E. coli* proteins, possibly as a result of dissipation of the proton motive force (35). In an initial experiment, in which we labeled the products of pKI175 maxicells in the presence of 6% ethanol, we observed partial inhibition of expression of the 25-kDa TraF band (data not shown). A subsequent experiment, in which *traF* expression from plasmids pKI175 and pKI272 was tested in the presence of 10% ethanol, is shown in Fig. 4. In this case, the ethanol markedly inhibited expression of the 29-kDa β-lactamase and 25-kDa *traF* product bands and caused the labeled 27-kDa *traF* product and 31-kDa β-lactamase precursor protein to accumulate. We concluded that the 27-kDa polypeptide (pre-TraF) is an unprocessed precursor of the 25-kDa TraF protein.

Cellular location of TraF protein. The cellular location of TraF was investigated by fractionating the periplasmic, membrane, and cytoplasmic proteins labeled in maxicells carrying pKI175, pKI272, or pKI280 prior to SDS-PAGE analysis. One such experiment with pKI175 is shown in Fig. 5A. Both pre-TraF and TraF protein bands could be detected

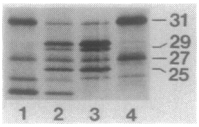

FIG. 4. Effect of ethanol on *traF* expression. Proteins labeled in maxicells carrying the TraF$^+$ plasmids pKI175 (lanes 1 and 2) or pKI272 (lanes 3 and 4). The presence of 10% ethanol (lanes 1 and 4) prevented the appearance of the 25-kDa TraF protein and the 29-kDa β-lactamase doublet. The 27-kDa and 31-kDa *traF* and *bla* precursor products accumulated in these samples.

in the membrane fraction. However, the periplasmic fraction clearly contained the most intensely labeled 25-kDa TraF protein band, and like β-lactamase, this TraF protein appeared to be concentrated in this fraction. In contrast, only faint bands corresponding to pre-TraF (27 kDa) and pre-β-lactamase (31 kDa) were detected in the periplasmic fraction.

The *trbA* (14.2 kDa) and *traQ* (12.5 kDa) products were enriched in the membrane fraction, as expected from our previous membrane fractionation results (46). Some additional pKI175 *tra* products could also be seen to distribute preferentially into the membrane fraction. Others, like the 25-kDa *traF* product, appeared to be most concentrated in the periplasmic fraction, indicating that additional *tra* operon products are also localized in the periplasm. Although most pKI175 *tra* products could also be detected in our cytoplasmic fraction, none appeared to be specifically enriched or concentrated in such samples. As the overall cytoplasmic radioactive polypeptide band profile resembled that of the membrane fraction, we suspect that the cytoplasmic profile reflects the presence of contaminating membrane material.

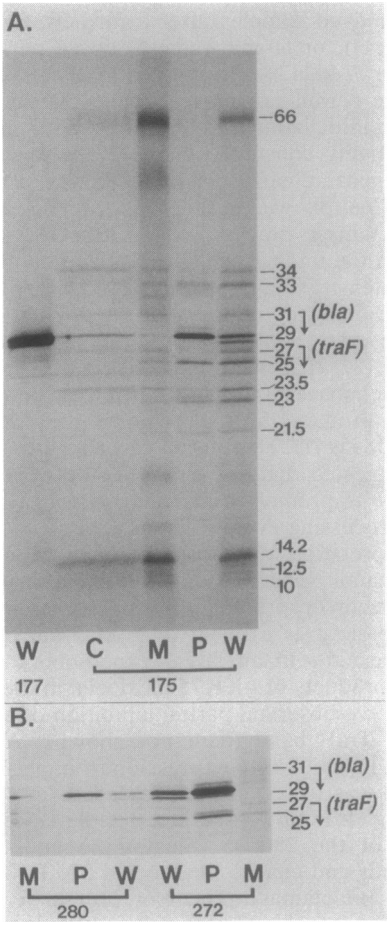

FIG. 5. Cellular location of *traF* products. (A) Plasmid pKI175 products were labeled in maxicells and whole-cell (W), periplasmic (P), membrane (M), and cytoplasmic (C) fractions were analyzed. A whole-cell sample of the pACYC177 control strain is also shown (lane W 177). (B) Central portion of a gel on which whole-cell (W), periplasmic (P), and membrane (M) fractions of labeled pKI280 and pKI272 products were analyzed. Numbers on the right indicate product size (in kilodaltons).

The relative intensity of *traF* bands found in cellular fractions derived from maxicells carrying pKI175, pKI272, and pKI280 was essentially the same (Fig. 5B); in each case, the 25-kDa *traF* product was enriched in the periplasmic fraction. Since pKI280 does not express *traQ*, this finding also demonstrated that processing and localization of the *traF* product are independent of the TraQ protein activity needed for efficient production of F-pilin polypeptide.

DNA sequence of *traF*. We determined the complete sequence of *traF* (Fig. 6). There was only one open reading frame in the sequence that crossed both the *Pst*I and *Hin*cII sites at nucleotides (nt) 441 and 740 previously mapped within *traF*; the *traF13* amber mutation was also known to lie between these sites, since clones carrying this region could recombine with F*lac traF13* to give plasmids with wild-type transfer properties (46). Therefore, we used DNA from plasmid pRS29*traF13* to construct an M13mp8 clone that carries the mutant *Pst*I-*Hin*cII segment and compared the sequences of *traF13* and wild-type F DNA. The *traF13* DNA sequence reflected a C → T transition mutation at nt 494 (Fig. 6) that altered a Gln codon to an amber codon. This confirmed the *traF* reading frame and placed *traF* between the in-frame TGA stop codons at nt 50 to 52 and 842 to 844. Although the first methionine codon in this frame was at nt 71, it was closely followed by several additional methionine codons (Fig. 6). Among these, only the fourth in-frame ATG (nt 100 to 103) was appropriately preceded by a region containing significant homology with the Shine-Dalgarno sequence (TAAGGAGG [39]). For this reason, we expect that translation of *traF* would actually initiate at the latter site, as suggested in Fig. 6. The *traF* translational product would then be a 247-amino-acid, M_r-28,006 polypeptide. The amino-terminal region of this polypeptide contained features similar to other leader peptides (43). These included a positively charged amino acid (Lys-3) followed by a stretch of predominantly hydrophobic amino acids ending in the sequence Pro-16–Ala-17–Ser-18–Gly-19. If peptide cleavage occurs between residues 19 and 20, a mature TraF protein of M_r 25,942 would result. Some structural predictions for the suggested 247-amino-acid pre-TraF polypeptide are shown in Fig. 7. The most distinctively hydrophobic segment was that in the amino-terminal signal region. Charged amino acids were otherwise distributed throughout the length of the *traF* product.

DISCUSSION

Our analyses of proteins labeled in maxicells demonstrate that the *traF* gene is expressed as 27-kDa polypeptide which is processed to a 25-kDa molecule. They further indicate that the majority of the TraF protein that is synthesized becomes localized in the cellular periplasm. Predictions based on the DNA sequence that we determined for *traF* are fully consistent with these findings. The gene product suggested is a 247-amino-acid, M_r-28,006 polypeptide that includes a 19-

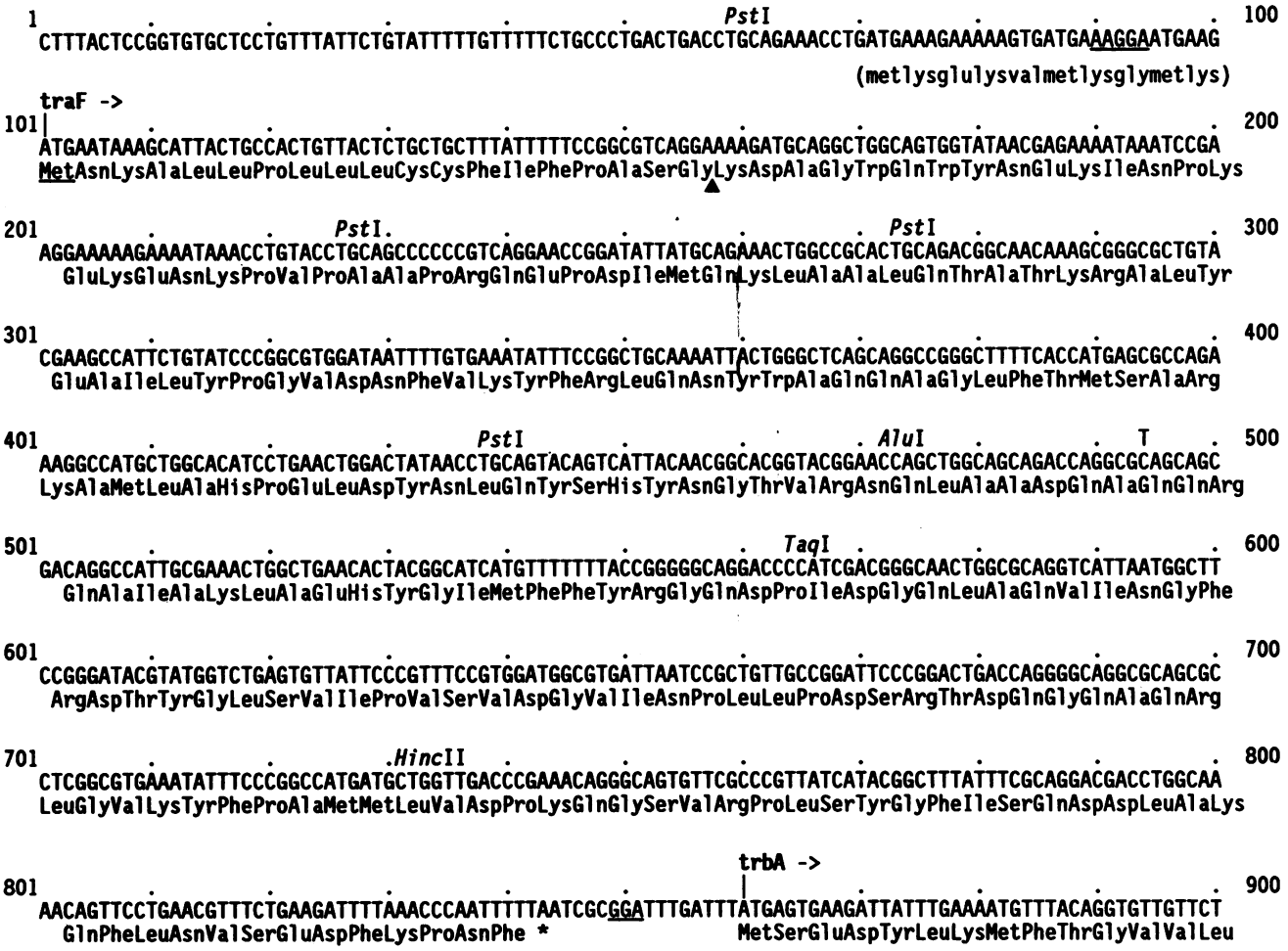

FIG. 6. DNA sequence of *traF*. The potential translational product of the entire 257-amino-acid open reading frame is shown. However, amino acids shown in parentheses seem unlikely to be translated. We suggest a 247-amino-acid *traF* product with a translational start site at nt 101, 7 nt distal to the underlined region that has homology to the Shine-Dalgarno sequence. A potential leader peptide cleavage site is indicated (▲). The C → T transition indicated at nt 494 is present in *traF13* mutant DNA. The beginning of the *trbA* open reading frame (Wu and Ippen-Ihler, manuscript in preparation) is also indicated.

amino-acid signal peptide leader sequence; removal of this signal peptide would result in a mature protein of M_r 25,942. While confirmation of the exact translational start and signal peptide cleavage site of the *traF* product will require purification and amino acid analysis of the *traF* protein products, the size of the predicted gene product is consistent with our protein data. The overall structure and composition of the predicted TraF protein also appear to be consistent with those of a periplasmic protein.

Although a product of *traF* was originally detected in outer membrane fractions obtained from minicells, periplasmic proteins were not examined in the previous study (27). We also detected *traF* products in membrane fractions. However, when we separated material from maxicells or λ*tra*-infected cells on sucrose density gradients, we detected only small amounts of the 25-kDa TraF protein in our pellets, and this product appeared to be concentrated in fractions of even lighter density than is characteristic for inner membrane material (24). When β-lactamase (29 kDa) was present, its distribution on the gradient paralleled that of mature TraF (data not shown). In the type of cell fractionation experiment reported here, we also found that the amounts of TraF and β-lactamase associated with the membrane pellet fraction

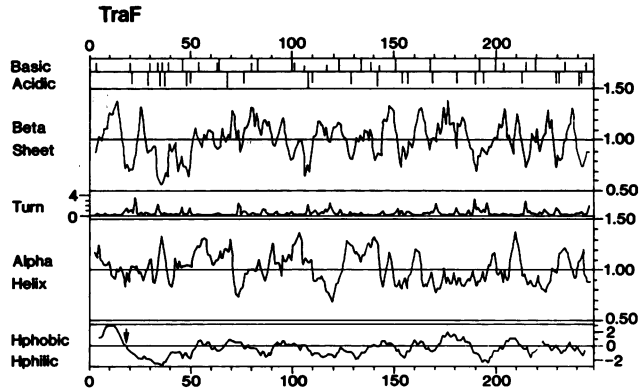

FIG. 7. Structural analysis of the predicted *traF* product. A portion of an analysis obtained with the University of Wisconsin Genetics Computer Group program PEPPLOT is shown. The distribution of charged amino acids throughout the molecule is indicated in the top panel. Secondary-structure predictions based on the method of Chou and Fasman (8) are shown in the three center panels as indicated. Hydropathicity (23) is graphed in the bottom panel; an arrow indicates the position of the leader peptide cleavage site proposed in Fig. 5.

were significantly reduced by the final wash and centrifugation step. Such results indicate that mature TraF is not an intrinsic membrane protein. The *traF* product is only the third *tra* operon product for which processing of a membrane signal sequence has been demonstrated. It is interesting that in each of these cases, the steps leading to production of the processed gene product appear to be different. In the case of *traA*, an unusually long signal peptide of 51 amino acids is present, and efficient production of the 70-amino-acid pilin polypeptide appears to require the action of an additional transfer operon product, TraQ (14, 33, 46). Furthermore, the pilin polypeptide appears to accumulate in the inner membrane unless additional *tra* proteins are active. The *traT* product, in contrast, is processed to an outer membrane lipoprotein; its signal peptidase cleavage site between residues 21 (Gly) and 22 (Cys) resembles that of other lipoproteins (20, 36, 43). Our data for *traF* indicate that this gene product is targeted to a third envelope compartment, the periplasm. As expression of the mature TraF was independent of *traQ* and no pair of Gly-Cys residues occurred in the *traF* translation product, it seems likely to be subject to the action of leader peptidase I (11). Preliminary studies of other *tra* gene products suggest that other *tra* proteins are similarly processed (J. Wu, P. Kathir, and D. Moore, unpublished data), and experiments such as that shown in Fig. 5 indicate that TraF is not the only periplasmic *tra* operon product. Experiments in progress in our laboratory are designed to characterize these additional proteins and protein-processing events and to determine whether a complex of pilus assembly proteins exists in the periplasm.

ACKNOWLEDGMENT

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