Location of an F-Pilin Pool in the Inner Membrane

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Polyacrylamide gel analysis of [^{35}S]methionine-labeled membrane preparations from *Escherichia coli* has revealed the presence of five polypeptides present only in the membranes of cells containing the conjugative plasmid F. In addition to the previously reported product of *traT*, polypeptides migrating with apparent molecular weights of 100,000, 23,500, 12,000, and 7,000 were resolved. Membrane preparations from F *traJ* mutants lacked these polypeptides, indicating that all of these proteins are *tra* gene products. The 7,000-molecular-weight polypeptide comigrated with unlabeled purified F-pilin protein. About 4 to 5% of the total radioactive label in whole membrane preparations was present in this polypeptide, indicating the existence of a substantial pool of membrane-associated F-pilin. The polypeptide could be extracted from whole membrane preparations with Triton X-100 and was found in the inner membrane fraction of membranes separated by sucrose density centrifugation.

Genetic analysis of the conjugative plasmid F has led to the identification of more than 20 F genes which are involved in the process of DNA transfer directed by F (for reviews, see references 19 and 14). The products of many of these genes have been identified as polypeptides synthesized in vitro or in minicells by chimeric plasmids (2, 17), or as transducing phage products in UV cell systems (16, 30).

Efforts to detect F transfer gene products in whole-cell preparations from *Escherichia coli* cells containing complete F factors have yielded poorer results. Only one major *tra* operon product, a protein involved in surface exclusion, has been reported (1, 21). F pili, the surface fimbriae elaborated by F, have also been purified and analyzed in several laboratories. These filamentous structures contain only one protein subunit, F-pilin (6, 10, 13, 15). The process by which F-pili become assembled is not yet known, but at least 13 genes must function before F-pili can be elaborated on the cell surface. Therefore, biosynthesis and assembly of these structures is clearly a complex process. Elucidation of the pathway which leads to the biosynthesis of F-pili has been complicated by the failure of previous analytical procedures to detect a pool of F-pilin in an unassembled state in the cell. Thus, gene products associated with synthesis of the polypeptide subunit have not been distinguishable from those which participate in its assembly into a pilus structure. In this work, we have analyzed membrane preparations from female and F*lac*-containing cells. Since the male cell

preparations, unlike those from hosts of chimeric plasmids or transducing phages, are derived from cells in which all F genes are active, we hoped to be able to identify proteins which might be the product of, or be affected by, several F gene activities. In particular, we sought to obtain a system whereby unassembled F-pilin could be identified in the membrane and thus facilitate study of the biosynthesis and assembly of this subunit.

MATERIALS AND METHODS

Bacterial strains and cultures. The female and male strians used in this investigation were JC3272 (F⁻ Δ*lacX74 gal his trp lys str tsx*) and its F*lac* derivative JC3273 (4). F *lac tra* strains are derivatives of JC3272 carrying F *tra* operon mutants, as described by Achtman et al. (4, 5).

Minimal medium for labeling with [^{35}S]methionine was a synthetic medium (11) containing glucose. It was supplemented with thiamine and all amino acids except cysteine and methionine.

Super broth contained, per liter, 32 g of tryptone, 20 g of yeast extract, and 5 g of NaCl.

Crude membrane preparations. Simple preparations of radioactively labeled whole membrane material were made essentially as described by Lutkenhaus et al. (18). Cells were grown to an optical density of 0.5 at 550 nm in minimal medium with vigorous aeration at 37°C. Usually, 30 μCi of [^{35}S]methionine (~4 × 10⁻⁴ μg) was added to 2 ml of cells and incubated for a further 5 min at 37°C. Cells were pelleted by low-speed centrifugation, suspended in 1.2 ml of Tris-EDTA buffer (10 mM Tris-hydrochloride–5 mM EDTA–1 mM dithiothreitol, pH 7.8), and sonicated for 105 to 120 s with the stepped microtip of a Heat

Systems cell disruptor (setting 2.8) until clear. After low-speed centrifugation to remove unlysed cells, the supernatant was centrifuged for 45 min in a Beckman SW50.1 rotor at 29,000 rpm. The membrane pellet was resuspended in the same Tris-EDTA buffer and centrifuged again at 29,000 rpm as before. It was then suspended in 100 μl of sample buffer for analysis on sodium dodecyl sulfate (SDS)-polyacrylamide gels (28).

Triton X-100 extraction of membrane preparations. Membranes which were to be extracted with Triton X-100 were prepared as described above, except that after the first high-speed spin, the membrane pellet was suspended in Tris-MgCl buffer (10 mM Tris–10 mM MgCl, pH 7.8) and incubated for 30 min at room temperature. This step was taken to avoid any partial extraction of outer membrane proteins which might occur in the absence of Mg^{2+} (27). The membranes were then pelleted by centrifugation for 45 min at 29,000 rpm as before and suspended with a 30-s burst of sonication in 2 ml of Tris–MgCl buffer containing 2% Triton X-100. The membranes were diluted to a final volume of 5 ml with the same Tris-MgCl-Triton X-100 buffer, allowed to stand for 15 min at room temperature, and then centrifuged at 45,000 rpm for 60 min in the SW 50.1 rotor. At this point the samples were separated into supernatant (Triton-soluble) and pellet (Triton-insoluble) fractions for further analysis. The Triton-insoluble pellet was resuspended and pelleted once more in Tris-MgCl buffer to remove residual Triton X-100 before final suspension in 100 μl of electrophoresis sample buffer (28) and gel analysis.

Separation of purified inner and outer membrane components. Small, radioactive quantities of inner and outer membranes were prepared as follows. Cultures (100 ml) were grown to an optical density of 0.5 at 550 nm in minimal medium. A 20-ml portion was removed to a separate flask to which 0.2 mCi of [^{35}S]methionine was added. Both cultures were allowed to continue growth for one more generation (50 min) and then centrifuged at room temperature. The labeled and unlabeled cell pellets were resuspended in a phosphate-free buffer according to the procedure of Ridgeway et al. (26) and pooled to make a total volume of 25 ml. Incubation at 37°C was continued for 50 min more, after which the cells were chilled and pelleted by centrifugation. All further steps were performed in the cold. The cell pellet was washed once with 25 ml of 30 mM Tris-hydrochloride (pH 7.8) and suspended in 7 ml of plasmolysis buffer as described by Osborn et al. (24). Lysozyme was added to a final concentration of 100 μg/ml, and the cells were allowed to stand for 2 min. Cold, 17 mM EDTA (pH 7.8) (14 ml) was added slowly over a period of 8 min. The spheroplasts formed were disrupted by sonication with the microprobe of the Heat Systems cell disruptor. Bursts (1-min) of sonication were continued until the culture was clear. Unlysed cells were removed by low-speed centrifugation, and the supernatant was then centrifuged for 90 min at 21,000 rpm in a SW27 rotor. The membrane pellet was resuspended in 2 ml of membrane suspension buffer (24), dialyzed overnight against 500 ml of 3 mM EDTA (pH 7.8), pelleted by centrifugation for 90 min at 26,000 rpm in the SW 50.1 rotor, and

suspended in 0.9 ml of 5 mM EDTA (pH 7.5) containing 25% sucrose. A 0.7-ml amount was then layered on a gradient consisting of layers of 0.5 ml of 55% sucrose and 0.7 ml each of 50, 45, 40, 35, and 30% sucrose in 5 mM EDTA (pH 7.5), and the gradient was centrifuged at 45,000 rpm for 18 h in the SW50.1 rotor. After fractionation of the resultant gradient, 1-μl aliquots were removed from each fraction for assay of radioactivity.

Polyacrylamide gel electrophoresis and autoradiography. SDS-polyacrylamide slab gel electrophoresis was performed as described by Studier (28) with a Bio-Rad model 221 dual vertical electrophoresis cell to form gels 280 by 140 by 0.75 mm. Separating gels were poured as an exponential gradient (mixing volume, 12.5 ml; total volume, 30 ml) of 10 to 16% acrylamide with a ratio of acrylamide:N,N'-methylene-bis-acrylamide of 30:0.8 in the top of the gradient and 30:1.6 in the bottom (25). All samples were heated in a boiling water bath for 3 min before loading on the gradient gel. Equivalent amounts of radioactivity (usually about 250,000 cpm) were normally loaded into each sample well. Electrophoresis was at 10 mA to a maximum of 200 V and was terminated when the bromophenol blue tracking dye migrated from the end of the gel. All gels were stained for 10 min in 0.15% Coomassie brilliant blue–45% trichloroacetic acid, diffusion destained in 7% acetic acid, and dried onto filter paper. Autoradiograms were made by exposure to Kodak SB-5 X-ray film.

Purification of F-pili. Small quantities of F-pili were purified by a modification of published procedures (15, 22). Cells were grown in 1 liter of super broth with vigorous aeration to an optical density of 1.7 at 550 nm. After 10 ml of 20% glucose was added, the cells were incubated for 30 min, and then chilled quickly by addition of ice and pelleted in a Sorvall GS1 rotor. The supernatant was set aside, and the cells were suspended in 0.05 M Na_2HPO_4 buffer (pH 7.1) and blended for three 15-s intervals in a Waring blender. The cells were removed again by centrifugation, and the blended supernatant was pooled with the original culture medium. Polyethylene glycol 6000 was added to the combined supernatant to a final concentration of 2% and NaCl was added to 0.5 M. Precipitation was allowed to occur overnight in the cold. The precipitate was then collected by centrifugation, dissolved in 20 ml of 80% sucrose in Tris-EDTA buffer (0.1 M Tris-hydrochloride–0.5 mM EDTA, pH 7.8) by stirring overnight, and then dialyzed against Tris-EDTA buffer for 3 days. By this time, a long string of F-pili pseudocrystals had formed in the dialysis tube. These were collected by centrifugation, and dissolved in 0.9 ml of 80% sucrose in Tris-EDTA buffer as before. CsCl, in the same Tris-EDTA buffer, was then added to give a final density of 1.207 g/cm^3, and the material was centrifuged for 24 h in the SW50.1 rotor at 45,000 rpm. The pilin band was collected by puncturing the side of the tube with a syringe. Analysis of this material on SDS-polyacrylamide gels showed that although traces of other proteins were present, most of the protein was F-pilin. We, therefore, used this preparation without further purification as an unlabeled protein marker on our gels.

Materials. SDS and other chemicals for slab gel

electrophoresis were purchased from Bio-Rad Laboratories. The following proteins were used as molecular weight markers (molecular weights within parentheses): β-galactosidase (116,000), purchased from Worthington Diagnostics, and catalase (60,000), ovalbumin (43,000), carbonic anhydrase (29,000), α-chymotrypsinogen (25,000), soybean trypsin inhibitor (21,500), myoglobin (18,400), β-lactoglobulin (16,900), cytochrome c (12,300), and bovine trypsin inhibitor (6,150), all purchased from Sigma Chemical Co. Lysozyme was obtained from Sigma Chemical Co., [^{35}S]methionine was from New England Nuclear Corp. and Triton X-100 was from Calbiochem.

RESULTS

Location of F-pilin in membrane preparations from male cells. We initially sought conditions of polyacrylamide gel analysis which would permit us to identify F-pilin subunits in our crude membrane preparations if such subunits were present in the membrane of male cells. For this purpose, we prepared a crude membrane fraction from male and female cells which had been labeled with [^{35}S]methionine and compared the patterns, on a variety of SDS-polyacrylamide gels, of polypeptides present in these fractions. We initially compared gels on which proteins in the molecular-weight range of 11,000 to 13,000 were well separated, because previous reports (6, 10, 13, 15) suggested that F-pilin would band in this region. However, when an unlabled F-pili preparation was used as a marker on these gels, the F-pilin consistently migrated near the gel front, in the same position as the heavily labeled band of lipoprotein present in both male and female membrane preparations. Therefore, we experimented with gels which might cause the migration of the F-pilin marker to differ from that of lipoprotein. This was most effectively accomplished when we used a gel formed in an exponential gradient of 10 to 16% acrylamide. A typical result of electrophoresis using such a gel is shown in Fig. 1. The position of unlabeled F-pilin marker protein (apparent molecular weight, 7,000) as obtained from the stained gel is indicated. The autoradiogram shows the migration pattern of labeled polypeptides present in our male and female crude membrane preparations. A heavily labeled band, present in the male but not in the female membrane preparation, comigrated with the F-pilin marker. We consistently found this band in membrane preparations from a variety of F-prime and Hfr strains, and we conclude that it corresponds to F-pilin.

Three other polypeptides, also found in the male membrane preparations only, are also indicated in Fig. 1. One, which was initially identified in our laboratory after examination of similar membrane preparations (21), has also
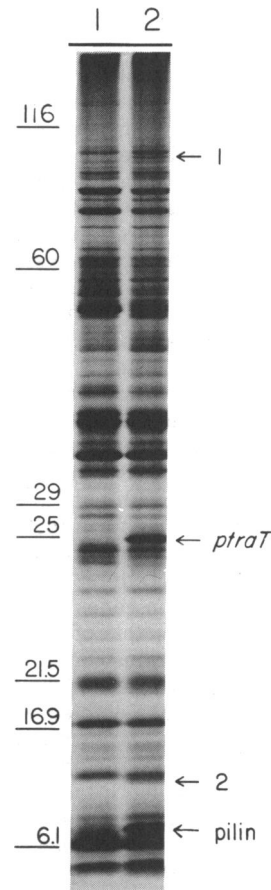

FIG. 1. *Analysis of membrane preparations from a male and female cell. Samples containing 250,000 cpm of [^{35}S]methionine-labeled protein were fractionated on a 10 to 16% exponential gradient of polyacrylamide as described in the text. A photograph of the autoradiogram of the finished gel is shown. Samples were loaded as follows: lane 1, crude whole membrane preparation from JC3272 (F$^-$); lane 2, crude whole membrane preparation from JC3273 (Flac). Bars indicate the positions, as obtained from the stained gel, of the molecular weight marker proteins which were coelectrophoresed in an adjacent slot. Arrows indicate the positions of proteins observed in lane 2 which are absent in lane 1. These include the traT product (ptraT). The (arrow labeled pilin also marks the position of purified nonradioactive pilin protein as obtained from the stained gel.*

been subsequently observed by several other laboratories and shown to be the product of the surface exclusion gene, *traT* (1). The two others, a polypeptide with an apparent molecular weight of 100,000 (Fig. 1, arrow 1) and a polypeptide with an apparent molecular weight of approximately 12,000 (Fig. 1, arrow 2), were present in much smaller quantities, and have

not previously been reported. As with F-pilin, we have consistently detected these proteins in other F' and Hfr membrane preparations; they were absent in all female strains tested. Some additional variations in band patterns between the female and male membrane preparations are also visible on this autoradiogram. For example, there are several bands which appear to be more intense in the preparation from female cells. These variations have, however, been found to be inconsistent in our membrane preparations, and may correlate with slight variations in culture and sample preparation, rather than with the presence of F plasmid.

Examination of membrane preparations from F *tra* gene mutants. It was important to determine whether the presence of F-pilin in our membrane preparations was due to the existence of a pool of F-pilin subunits in the membrane, or resulted from the fortuitous association of free F-pili with the membrane fraction during our preparation procedure. A number of mutations of F *tra* operon genes are known to cause the host to be incapable of elaborating F-pilus filaments. These strains could not produce free F-pili, but most of them might be expected to contain pools of unassembled F-pilin subunits. Therefore, we chose two such mutants for study and analyzed crude membrane preparations from cells containing F'*lac* plasmids bearing either a *traE* or a *traF* mutation. To determine whether the F-specific proteins we were observing derived from the *tra* operon of F, we also examined a membrane preparation from a strain in which the F*lac* plasmid contained the *traJ90*(Am) mutation. This mutation causes deficient expression of all *tra* operon products since the *traJ* product is required for *tra* operon transcription (29). The analysis of these membrane preparations is shown in Fig. 2.

An F-pilin band is present on the autoradiogram of membrane polypeptides prepared from the F*lac* derivatives containing the *traE* and *traF* mutations. Therefore, although these strains are unable to produce functional or morphologically identifiable F-pili, they do appear to make F-pilin subunits which are resolved on our gels. We can conclude that our method of analysis does assay the presence of F-pilin subunits which exist unassembled in the membrane. The appearance of the other F-specific bands we observed on these gels was also unaffected by the *traE* or *traF* mutations. Therefore, eliminating these gene products does not affect the appearance of these polypeptides in the membrane. Examination of the membrane preparation from the *traJ* mutant shows, in contrast, that the F-specific proteins migrating as 100,000-dalton and 12,000-dalton bands (Fig. 2, arrows 1

and 2, respectively), as well as F-pilin, are missing from this preparation. Therefore, all of these polypeptides appear to be *tra* operon products. As has been noted elsewhere, the *traT* product band is diminished in intensity but not absent in the *traJ90* mutant membranes (3). On this particular gradient gel, the *traT* product is not completely resolved from a band also present in the female membrane preparation. Usually, these two bands migrate at slightly different positions.

Examination of membrane preparations after extraction with Triton X-100. Since Triton X-100 has been reported to solubilize cytoplasmic membrane components preferentially (27), we examined the effect of extracting our membrane preparations with this detergent. Analysis of the Triton-insoluble proteins which remained in our preparations after extraction with 2% Triton X-100 is also shown in Fig. 2. It is clear that the F-pilin band has been lost as a result of this extraction. Thus, the F-pilin subunits are behaving as inner membrane components. The *traT* product is unaffected by the extraction procedure, as would be expected from its designation as an outer membrane component (1, 19, 21). The 100,000-dalton protein appears to be solubilized by this procedure, although a trace of this band can still be seen on the autoradiogram. The F-specific 12,000-dalton protein is still very evident. Small amounts of another F-specific protein (Fig. 2, arrow 3) are also visible in the Triton-insoluble preparation from the male and *traE* and *traF* membranes, but not in the female and *traJ* Triton-insoluble preparations. It runs very close to a protein which is present in the female and male whole membrane, and is thus obscured in the whole membrane preparations analyzed on this gradient.

We were unable to analyze Triton-soluble proteins because our Triton X-100 extract was too dilute. Analysis of trichloroacetic acid precipitates of the extract were precluded by the small amount of protein in our radioactive membrane preparations. They contained such a high ratio of detergent to protein that the bands were a smear on SDS-polyacrylamide gels.

Examination of separated inner and outer membrane preparations. To determine by an alternative method whether the proteins we were observing were associated with the inner or outer membrane of the host cell, separation of radioactively labeled inner and outer membrane components was performed on isopycnic sucrose density gradients, as described above. Profiles of the gradient obtained from the female and male strains are shown in Fig. 3. Peak outer and inner membrane fractions were
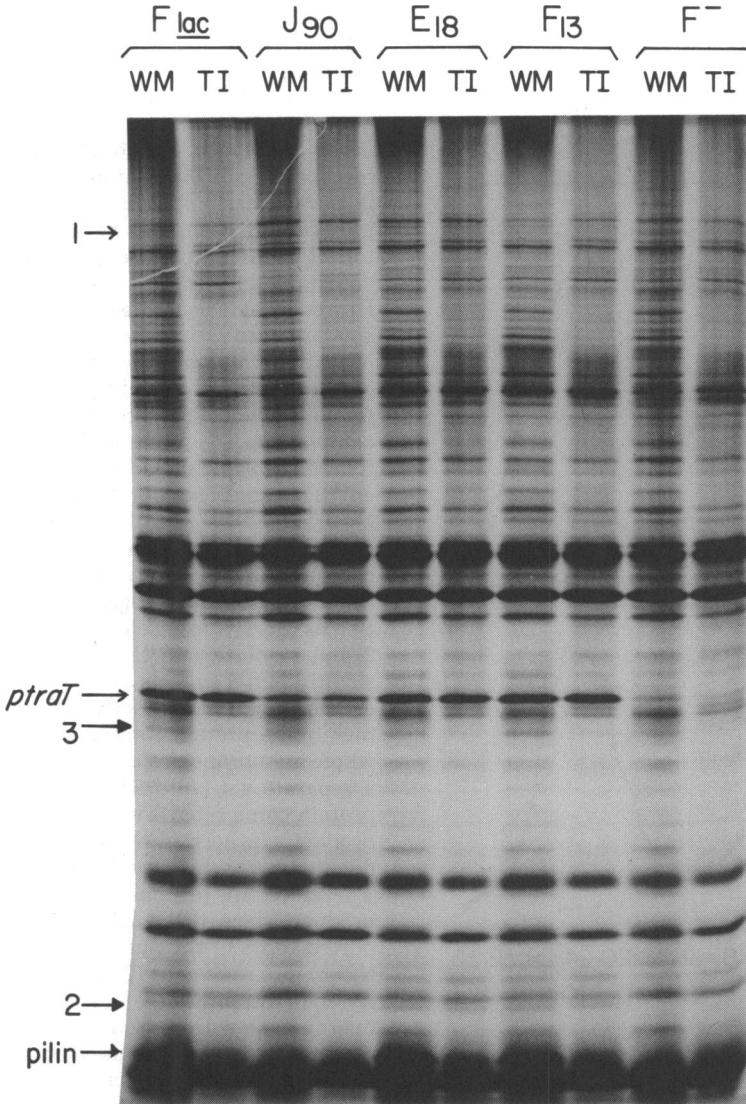
FIG. 2. *Analysis of crude whole membrane (WM) and Triton insoluble membrane (TI) preparations from Flac and Flac tra mutant hosts. Polyacrylamide gel analysis was performed on membrane preparations from strains carrying Flac, Flac traJ90, Flac traE18, Flac traF13, or no plasmid (F⁻). For each strain indicated at the top of the figure, a WM preparation and the TI fraction obtained after extraction of the membranes with 2% Triton X-100 were examined. 200,000 cpm of radioactive protein was loaded in each sample well. Arrows indicate the location of the F-specific proteins traT product (ptraT), F-pilin, the 100,000-dalton protein (arrow 1), and the 12,000-dalton protein (arrow 2) as noted in the legend to Fig. 1. An additional F-specific protein (arrow 3) can be distinguished in the Triton-insoluble fraction of membranes from the Flac, Flac traE18 and Flac traF13 strains.*

pooled as indicated, pelleted by centrifugation, and suspended in sample buffer for SDS-polyacrylamide gel analysis. The result of this analysis is shown in Fig. 4.

It is obvious from the autoradiogram shown that F-pilin is present in significant quantities in the inner membrane fractions of the male cell membrane preparation; it is not visible in the outer membrane preparation. Since the specific activity of these membrane preparations was less than that of our crude membrane preparations, overloading of protein might have made detection of pilin difficult in this channel of the autoradiogram shown; however, pilin was also
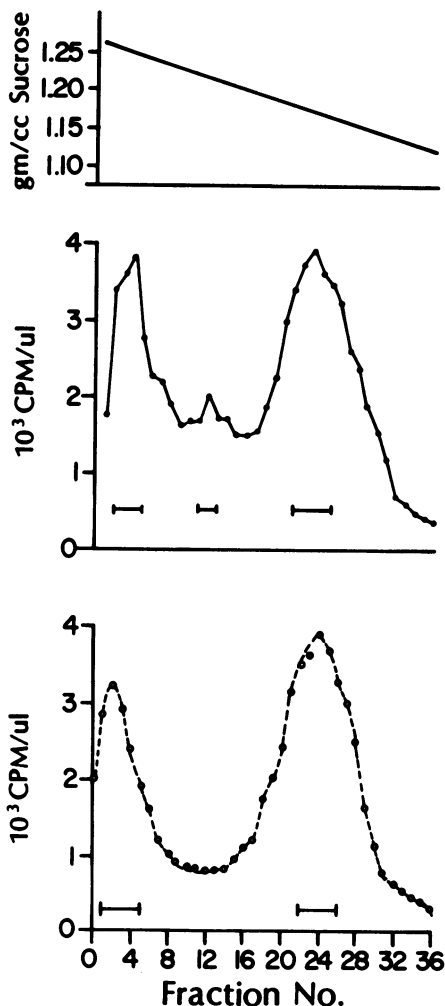
FIG. 3. *Separation of inner and outer membrane fractions. [^{35}S]methionine-labeled whole membrane preparations were prepared and subjected to sucrose density centrifugation as described in the text. Density of sucrose as calculated from the refractive index is shown in the graph at the top. Counts per minute per microliter of fractions from the gradients of the male strain, JC3273 (●), and the female strain, JC3272 (○), are indicated in the middle and bottom figures respectively. Bars indicate fractions which were pooled and concentrated for further analysis.*

not visible when smaller quantities of outer membrane material were analyzed (data not shown). The quantity of lipoprotein present in the whole membrane preparation did cause the slight difference in position observed between the pilin band in the whole membrane and the inner membrane channels on this gel. This artifact was not observed when smaller samples were analyzed. In conjunction with the Triton

X-100 experiments, our results indicate that F-pilin is associated with inner membrane components.

The 100,000-dalton protein (Fig. 4, arrow 1) appears to be present in small quantities in both inner and outer membrane prepared by this method. The 12,000-dalton protein (Fig. 4, arrow 2) is also clearly present in this inner membrane preparation. Although we had found this band to act as a Triton-insoluble protein, it is not detectable in this outer membrane preparation. However, as this protein is usually present in small quantity, it would have been obscured by other outer membrane components in this region of the gel. *traT* protein product, as has been found previously, is clearly an outer membrane component, although small quantities of a protein of the same molecular weight are still present in our inner membrane fraction. At a slightly lower molecular weight than that of the plasmid *traT* product, we again see an additional F-specific protein (Fig. 4, arrow 3) in the outer membrane fraction. It is migrating in the same position as the Triton-insoluble protein noted in Fig. 2, but resolution in this region of the gel was slightly better on this gradient, and the protein can be seen to be distinctly different from female membrane protein components even when the whole membrane preparations are compared. In a separate gel (autoradiogram not shown) this protein comigrated with the 23,500-dalton *traJ* product synthesized by the lambda *traJ* transducing phage which we previously reported (16). Therefore, this protein may be the plasmid *traJ* product, since the *traJ* product has been reported to be in the outer membrane when synthesized in mini cells (2). However, the possibility that the protein is encoded by another *tra* operon gene with a product of the same molecular weight is not precluded, since such a product would also be expected to be absent in the membranes of *traJ* mutants.

In Fig. 3 it can be seen that membranes from the male but not the female strain also contained material which banded at an intermediate density on the sucrose gradient. We also pooled material in this region (fractions 11 to 13 of the male sucrose gradient) and examined it. The protein profile resembled our whole membrane preparation, although outer membrane proteins were somewhat more pronounced. All the F-specific proteins were very evident in this fraction, suggesting to us the possibility that F *tra* gene proteins may be enriched in such intermediate density material. We are examining this hypothesis further.

Quantity of pilin in the membrane. Estimates of the relative amount of F-pilin present in our membrane preparations were made by

scanning our autoradiograms with a Joyce Loebl Mark II densitometer, and integrating the tracings of the peaks obtained with a Numonics graphic calculator. These analyses showed that approximately 4 to 5% of the total radioactivity present in our crude membrane preparations was associated with F-pilin. In comparison, 3 to 4% of the total radioactivity was associated with the other major F-specific protein, the product of traT. A gel comparable to that shown in Fig.

1 was also prepared and dried in the absence of stain and destaining procedures. Bands which appeared on the autoradiogram of this gel were cut out through the X-ray film, eluted in 0.2% SDS and assayed for radioactivity. Of the total counts applied in the male membrane sample well, 3.8% could be eluted from the F-pilin band, 2.8% from the traT product band, and 4.2% from the band corresponding to the major membrane protein II*.

DISCUSSION

Our primary goal in this analysis was to detect pilin subunits in the membrane in order to develop an assay for the presence of F-pilin in the membranes of mutants which do not elaborate F-pili filaments. Our conclusion that we have, indeed, detected these subunits is based upon the comigration of an intensely labeled F-specific polypeptide with purified F-pilin. That this band is a tra operon product is substantiated by its absence in the membrane of traJ mutants. We can further conclude that the polypeptide we see does not result from the contamination of our membrane preparations with free F-pili, because the same Triton X-100-extractable protein is visible in the membranes of Ftra mutant cells which do not synthesize F-pili filaments. Subsequent experiments have shown that membranes of FtraA mutants do not have this polypeptide (D. Moore, B. A. Sowa, and K. Ippen-Ihler, manuscript in preparation). This is consistent with our identification, since traA mutations are known to affect pilin structure (22, 31).

The molecular weight of F-pilin has been reported to be 10,750 to 12,100 (6, 10, 13, 15). In these studies, the minimum molecular weight of F-pilin as deduced from its amino acid composition has been doubled to agree with apparent molecular weight values obtained by SDS-polyacrylamide gel analysis. However, F-pilin has migrated very close to the front of the gel systems used. We have found that F-pilin, in the membrane, is not separated from other membrane components unless better resolution in the low-molecular-weight region of the gel is obtained. Under our conditions, F-pilin migrated at an apparent molecular weight of about 7,000, well ahead of a 12,300-dalton marker (cyto-
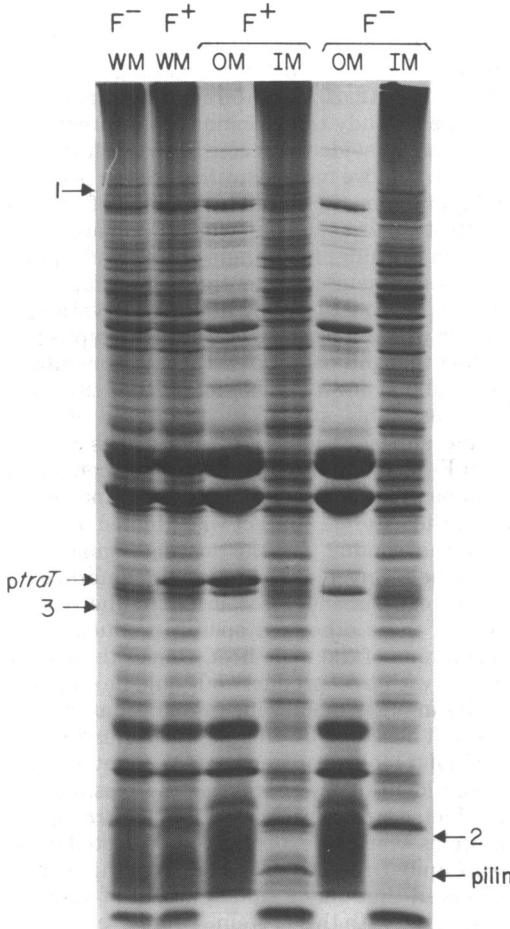

FIG. 4. *Analysis of separated inner and outer membrane fractions. Samples of whole and separated membrane preparations containing 500,000 cpm of [35S]methionine-labeled protein were analyzed by polyacrylamide slab gel electrophoresis as indicated previously. The first two lanes show protein present in the whole membrane (WM) preparations from the female (F⁻), JC3272, and the male (F⁺), JC3273, strains used. These are compared with the proteins present in the outer (OM) and inner (IM) male and female membranes after fractionation by sucrose density centrifugation. The latter preparations were obtained by pooling fractions 2 through 5 (F⁺, OM) and 21 through 25 (F⁺, IM) of the JC3273 gradient and*

fractions 1 through 5 (F⁻, OM) and 22 through 26 (F⁻, IM) of the JC3272 gradient as indicated in the legend to Fig. 3. Arrows indicate the positions of bands corresponding to the four F-specific proteins identified in Fig. 1: band 1 at 100,000 daltons, band 2 at 12,000 daltons, traT product (ptraT), and F-pilin. Arrow 3 indicates a faint F-specific band which in a separate gel was shown to migrate at the same position as the product of traJ.

chrome c), and just behind the bovine trypsin inhibitor (6,150 daltons). This raises the possibility that the pilin monomer polypeptide could have an actual molecular weight of about 6,000. However, this minimum molecular weight would predict only one tyrosine residue per molecule, whereas Minkley et al. (22) have found two different tyrosine containing tryptic peptides. Therefore, we believe that the apparent molecular weight we obtain in our gel system is low, and an artifact which results from a peculiarity of the pilin molecule. Armstrong et al. (6) have reported that SDS only slightly decreased the α-helicity of purified F-pilin; retention of secondary structure even in the presence of SDS could easily explain our result. Since purified F-pilin has been reported to contain sugars and phosphate (6, 10, 13, 15), and may be tightly associated with phospholipid even on the SDS gel (W. Paranchych, personal communication), other explanations for the anomalous position of F-pilin on our gels are also possible.

The finding that our F-pilin band could be extracted from whole membrane preparations with Triton X-100 and was associated with inner membrane preparations was, at first, surprising to us. Purified F-pili are not soluble in Triton X-100, presumably because pilin subunits are not dissociated by this detergent (15). The report by Beard and Connolly (8) that a protein resembling R1drd-19 pilin was in the outer membrane of hosts containing this plasmid had suggested to us that we would also find F-pilin to be an outer membrane protein. We are presently investigating whether these results reflect a fundamental difference in the way F and R1 pili are synthesized, or whether they stem from procedural differences, such as our use of log-phase cells rather than stationary-phase cells (which were employed in the R1drd-19 experiments).

Inability to detect F-pilin in membrane preparations led Manning and Achtman (19) to suggest that a pool of mature pilin did not usually exist, but instead might be present only in a higher-molecular-weight precursor form. One difficulty with this suggestion has been that the higher-molecular-weight precursor has also not been evident in any significant quantity in analysis of the membranes of male cells, or in mutants blocked in pilus production. Yet, a large number of pilin subunits are needed to elaborate an F-pilus filament, and F-pilus regeneration studies have indicated that a pool of presynthesized protein was available for assembly into these filaments (9). Our results show that a membrane pool of pilin polypeptide does in fact exist. The protein that we have resolved is rapidly labeled and synthesized in quantities comparable to the major membrane proteins. It is

also interesting to note that the polypeptide in this pool did not turn over rapidly, since no significant difference in the relative quantity of protein present was evident in our experiment with purified membrane components in which cells had been incubated for a generation after labeling. In other studies we have found that pilin polypeptide labeled at mid-log phase could still be detected in preparations from cells grown to late-stationary phase. This may imply that the pilin pool is large relative to the number of F-pili which are produced by the cells, or that F pilin is not necessarily lost after F-pilus formation, but returns to the membrane by F-pilus retraction (12, 20, 23). More quantitative experiments to study this question are in progress.

Thus, all evidence is consistent with the conclusion that we have identified a large pool of F-pilin in the membrane. The location of this pool suggests to us a model in which F-pili are assembled in the inner membrane and extend through the outer membrane to the cell surface. This would be consistent with the observations of Bayer who obtained electron micrographs indicating that pili penetrated the outer membrane in a tubular form (7). He found that sites of F-pilus assembly appear to be located at membrane adhesion zones (7). Thus, it is possible that F-pili might provide a focus for a connection between the inner and outer membrane, and be both the external contact with the recipient cell and the conduit for the mating signal which must be received at the internal membrane.

Our experiments also permitted the recognition of three other tra operon products in the membrane of the male host. One of these comigrated with traJ product and could be seen most easily in outer membrane preparations where it was not obscured by an inner membrane protein of similar molecular weight. We cannot unequivocally identify the protein as the plasmid traJ product, since expression of other tra operon proteins would be expected to be affected by the traJ amber mutation. It is interesting to note, however, that only a small amount of label was associated with this protein. Therefore, the plasmid traJ product cannot be present in quantity. The membrane location of the two other tra operon products we observe is somewhat ambiguous. Both were easily detectable in the inner membrane fraction we purified on the basis of density in sucrose. Trace amounts of the 100,000-dalton polypeptide were, however, also detectable in the outer membrane fraction prepared in this way, and both the 100,000- and the 12,000-dalton proteins were observable in membrane preparations which had been extracted with Triton X-100. It is possible that these polypeptides are associated with both the outer and the inner

membrane. The identity and function of these proteins are being investigated. Analysis of additional *tra* mutants indicates that the 100,000-dalton protein is the product of *traG* (Moore, Sowa and Ippen-Ihler, manuscript in preparation).

LITERATURE CITED

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