Analysis of the *traLEKBP* Sequence and the TraP Protein from Three F-Like Plasmids: F, R100-1, and ColB2

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The sequence of a region of the F plasmid containing the *traLEKBP* genes involved in plasmid transfer was compared to the equivalent regions of two IncFII plasmids, R100-1 and ColB2. The *traLEK* gene products of all three plasmids were virtually identical, with the most changes occurring in TraE. The *traB* genes were also nearly identical except for an 11-codon extension at the 3′ end of the R100-1 *traB* gene. The TraP protein of R100-1 differed from those of F and ColB2 at its N terminus, while the ColB2 TraP protein contained a change of sequence in a predicted loop which was shown to be exposed in the periplasmic space by Tn*phoA* mutagenesis. The effect of the altered TraP sequences was determined by complementing a *traP* mutant with clones expressing the *traKBP* genes of F, R100-1, and ColB2. The *traP* mutation in pOX38 (pOX38-*traP474*), a derivative of F, was found to have little effect on pilus production, pilus retraction, and filamentous phage growth and only a moderate effect on transfer. The transfer ability of pOX38-*traP474* was shown to be affected by mutations in the *rfa* (lipopolysaccharide) locus and in *ompA* in the recipient cell in a manner similar to that for the wild-type pOX38-Km plasmid itself and could be complemented with the *traP* analogs from R100-1 and ColB2 to give an F-like phenotype. Thus, the TraP protein appears to play a minor role in conjugation and may interact with TraB, which varies in sequence along with TraP, in order to stabilize the proposed transmembrane complex formed by the *tra* operon products.

The genes involved in establishing an effective mating pair during F plasmid-mediated conjugation are encoded within the 33-kb transfer (*tra*) operon. Fifteen of the nearly 40 known genes in the *tra* operon are involved in F pilus synthesis and assembly (for a review, see reference 7). This filamentous extracellular organelle is thought to identify suitable recipient cells by binding to a receptor on the cell surface and drawing the recipient cell to the donor cell surface via pilus retraction. The process of retraction is controversial and is based on electron microscopic examination of cells during mating pair formation and filamentous phage (f1, M13, or fd) infection. In addition, a second role for the F pilus involving the actual transfer of DNA has been proposed on the basis of evidence of DNA transfer between cells separated physically from one another but connected by a pilus (11). This latter function for the F pilus is in agreement with genetic analysis of the F *tra* region, since mutations in genes that affect pilus synthesis block DNA transfer while mutations in the recipient cell which decrease mating pair formation have only a modest effect (2 logs) on transfer efficiency.

Mating pair formation by the F plasmid has been shown to require OmpA and specific moieties within the inner core of the lipopolysaccharide (LPS) in the recipient cell. The pilus subunit is probably not involved in recognizing these structures, nor is it involved in recognition of the TraT surface exclusion protein, which reduces nonproductive mating between donor cells (2). This suggests either that a minor component of the pilus is located at the tip and acts as an adhesin or that other *tra* proteins, especially TraN in the outer membrane, are involved in interacting with LPS (or OmpA) during mating pair formation.

The genes involved in pilus synthesis in closely related F-like plasmids are able to complement mutations in the analogous gene in the F plasmid (25), and the DNAs of these plasmids have been shown to be highly homologous by heteroduplex mapping of the entire *tra* region (23). Differences in the protein sequences of the gene products among these F-like plasmids tend to be clustered in small domains, suggesting specificity of function (7). We are interested in determining whether these small differences in sequence can be related to differences in mating pair formation and pilus function (e.g., pilus retraction and f1 phage attachment) among three F-like plasmids, F*lac* (F), R100-1*drd* (R100-1), and ColB2-K77 (ColB2) (14, 25). Unlike F and ColB2, R100-1 does not require OmpA in the recipient cell and does not recognize substituents in the inner core of the LPS, although it may require specific side groups in the 2-keto-3-deoxyoctulosonic acid–lipid A region (2). It is also highly resistant to f1 phage (6). While the F mating system appears to require the pyrophosphorylethanol-amine (PPEA) group on heptose I in the LPS, ColB2 mating is affected by mutations that block addition of heptose 3 to heptose II in the inner core. Also, ColB2 pili do not retract in the presence of 0.01 M cyanide, nor do they confer sensitivity to f1 phage (6). These clues to the mechanism of pilus function and mating pair formation will be exploited in order to begin to assign functions to the many genes in the *tra* operon.

In this report, the sequence of the *traLEKBP* region of F is compared to those of the same regions in the F-like plasmids ColB2 and R100-1 (IncFII). Analysis of the sequences of the *traP* genes from the three F-like plasmids revealed precisely delineated differences in amino acid sequence. A derivative of pOX38 with a kanamycin resistance cassette inserted in the *traP* gene (pOX38-*traP470* or *-474*) had reduced levels of DNA transfer and lowered sensitivity to pilus-specific phages but produced pili that were longer than usual. Such characteristics might be expected of a mutation in a pilus tip protein which might affect pilus length determination, recipient cell recogni-

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TABLE 1. Bacterial strains used in this study

E. coli strain	Relevant genotype	Source or reference
CC118	Δ(*ara leu*)7697 *lac*Δ*X74* Δ*phoA20 galE galK thi rpsE rpoB argE*(*am*) *recA1*	C. Manoil
CS180-2	*thr leuB6 proA argE his thi galK lacY trpE mtl xyl ara-14 rpsL* Su⁺	C. Schnaitman
CS1977	Δ*rfaGI1*::ΩCmʳ of CS180-2 λSG3.4(*rfaQ⁺G⁺*)	C. Schnaitman
CS1981	Δ*rfaGI1*::ΩCmʳ of CS180-2 λSA4.9(*rfaQ⁺G⁺P⁺*)	C. Schnaitman
CS2057	Δ*rfaGI1*::Cmʳ of CS180-2 *cps-5*::Tn*10*	C. Schnaitman
CS2058	CS2057 but *rfaQJ2*	C. Schnaitman
JC3272	F⁻ *lac*Δ*X74 gal his trp lys rpsL tsx* (λ)	Lab stock
MC4100	F⁻ *araD139* Δ(*argF-lac*) *U169 rpsL150 relA1 flbB3501 deoC1 ptsF25 rbsR*	C. Manoil 2
XK1200	F⁻ *lac*Δ*U124* Δ(*nadA aroG gal attl*λ *bio*) *gyrA*	K. Ippen-Ihler
JC3051	F⁻ *lac*Δ*X74 his trp rpsL tsx mal* (λ)	Lab stock

tion, or f1 phage attachment. In light of the sequence differences in the TraP proteins of the F-like plasmids and the differences these plasmids exhibit in the requirement of receptors on recipient cells for conjugation (2) and in their patterns of phage attachment (6), TraP seemed to be a promising candidate for an adhesin at the pilus tip.

MATERIALS AND METHODS

Bacterial strains and plasmids. All *Escherichia coli* strains were cultured in liquid Luria-Bertani (LB) medium, and appropriate antibiotics were added at the following concentrations: ampicillin (Amp), 50 μg/ml; kanamycin (Km), 30 μg/ml; tetracycline (Tc), 20 μg/ml; chloramphenicol (Cm), 25 μg/ml; streptomycin (Sm), 200 μg/ml; and nalidixic acid (Nal), 40 μg/ml. Bacterial strains and plasmids used in this study are listed in Tables 1 and 2, respectively.

Mating assays and determination of mating efficiency profiles. Donor and recipient bacteria were grown in LB broth at 37 or 30°C to mid-log phase (an optical density at 600 nm of 0.4 to 0.6). At mid-log phase, 100 μl of each (donor and recipient) cell culture was added to 1 ml of fresh LB broth, and mating was allowed to proceed for 60 min at either 37 or 30°C. The mating was interrupted by vigorous vortexing followed by chilling on ice. Dilutions were made in ice-cold 1× SSC (0.15 M NaCl plus 0.015 M sodium citrate), pH 7.0 (8), and 10 μl of each dilution was spotted onto selective LB agar. The number of donor cells in each mating mixture was determined concurrently. The efficiency of mating was recorded as the number of transconjugants per 100 donor cells, and the overall proficiency of mating relative to that of a wild-type control was expressed as a percentage as reported earlier (2).

DNA sequence determination and analysis. The DNA sequences of the F plasmid *traL*, -*E*, -*K*, -*B*, and -*P* genes have been previously determined (7). A series of clones were constructed in M13mp18 and -19 (26) by using the *Hinc*II, *Sma*I, and *Pst*I sites in the *traLEKBP* region of ColB2 and the *traLE* region of R100-1. The *traLEKB* genes of ColB2 were sequenced by using the dideoxy chain-termination method of Sanger et al. (21) and the Klenow fragment of *E. coli* DNA polymerase I (Boehringer Mannheim). The *traP* genes from the ColB2 and R100-1 plasmids were amplified by PCR with Vent DNA polymerase (New England Biolabs) and the synthetic oligonucleotide primers KANB1 (5′-CGCC CAAGCTTGGGCAGTTTATGTCCGGCAGCC-3′) and LFR36 (5′-GAATTC TTCATCACACCGACAG-3′), which carry *Hind*III and *Eco*RI restriction endonuclease sites, respectively. The 770-bp (ColB2) and 790-bp (R100-1) fragments containing the *traP* genes were cloned into pUC118 (24) and subsequently sequenced by using the dideoxy chain-termination method (21) and Sequenase

version 2.1 supplied as a kit (U.S. Biochemicals). Primers KANE2 (5′-CGCG GATCCGCGCTGATATCAAACATTACATCCTTA-3′) and LFR36 were used to amplify the *traK* and *traB* genes from R100-1 in a PCR reaction, and the DNA fragment was cloned and sequenced as previously described by using the *Bam*HI and *Eco*RI sites provided in the primers. The DNA sequences were analyzed by using PC/GENE (IntelliGenetics). The DNA sequences were aligned with the program CLUSTAL by the method developed by Higgins and Sharp (12). Secondary-structure predictions for proteins were made by using the Garnier Robson algorithm (10).

Generation of *traP* gene fusions by Tn*phoA* transposition. Fusion of the *E. coli* alkaline phosphatase (AP) gene, *phoA*, to the *traP* gene was carried out with the Tn*phoA* delivery system described by Manoil (16). *E. coli* CC118 host cells transformed with pKI250 were grown at 37°C in liquid LB medium containing 10 mM MgSO₄ and 20 μg of tetracycline per ml. At early stationary phase, the cells were infected with λTn*phoA* at a multiplicity of infection of approximately 1.0. Procedures for transposition and outgrowth were as described by Manoil (16). Transpositions into pKI250 were initially selected on solid LB medium containing 300 μg of kanamycin and 40 μg of 5-bromo-4-chloro-3-indolylphosphate toluidinium (BCIP; Boehringer Mannheim) per ml but lacking tetracycline. Isolated colonies were replica plated onto solid LB medium containing 30 μg of kanamycin, 20 μl of tetracycline, and 40 μg of BCIP per ml. Plasmid DNAs from a large number of blue, pale-blue, and white colonies were isolated (20) and digested with *Eco*R1 restriction enzyme (Boehringer Mannheim) in order to identify specific transpositions into the *traP* gene. Candidates carrying such transpositions were further sequenced in order to determine the site of Tn*phoA* insertion by using a synthetic primer (5′-AATATCGCCCTGAGCA-3′) which hybridizes to the 5′ end of the *phoA* gene in the transposon and directs DNA polymerization through the transposon's left ends into the *traP* gene (16).

Construction of pOX38 derivatives. The construction of pOX38 derivatives took advantage of the unique *Pst*I site in the *traP* gene (7). The plasmid pKI468 (Table 1) was digested with *Pst*I and was ligated with a kanamycin resistance gene cassette (*kan*) containing *Pst*I ends derived from the parent plasmid pUC4KISS (15). Potential clones were selected on media containing ampicillin and kanamycin. The orientation of the kanamycin cassette was determined by restriction mapping. Two recombinant plasmids carrying the *kan* gene oriented either in the direction opposite of *traP* (pKI470) or in the same direction as *traP* (pKI474) were isolated. To obtain pOX38 derivatives, triparental matings were performed by mixing the donor strain, *E. coli* RD-17/pOX38 (18), with a derivative of strain XK5456 (18) carrying either pKI470 or pKI474 and with a final recipient strain, XK1200. After allowing in vivo recombination to occur, transconjugants were selected for kanamycin and nalidixic acid resistance and ampicillin sensitivity. Potential constructs were screened for the presence of the *kan* insert in pOX38

TABLE 2. Plasmids and transposons used in this study

Plasmid	Description of construct	Source or comment
pKI250	2.55-kb *Hinc*II fragment of F carrying *traPVR* gene of pBR322 (Tcʳ)	This work
pKI468	2.55-kb *Hinc*II fragment (as in pKI250), cloned into pKI497 (Ampʳ)	This work
pKI470	*kan* fragment from pUC4KISS inserted into *Pst*I site of *traP* in pKI468; *kan* is in anti-*tra* orientation	This work
pKI474	Same as pKI470, except *kan* is in same orientation as *traP*	This work
pKAF2	3.2-kb fragment containing *traK-traP* from F, cloned in pUC118 (Ampʳ)	This work
pKAR2	3.2-kb fragment containing *traK-traP* from R100-1, cloned in pUC118 (Ampʳ)	This work
pKAB2	2.9-kb *Eco*RV-*Eco*RI fragment containing *traK-traP* from ColB2 in pUC118 (Ampʳ)	This work
pOX38-Km	Tra⁺ RepFIA⁺ Kmʳ f1 *Hind*III fragment of F plus *Hind*III fragment of Tn*5*	3
pOX38-*traP474*	Tra⁺ RepFIA⁺ Kmʳ *kan* cassette inserted into *Pst*I of *traP*	This work
pOX38-*traP470*	*kan* cassette in orientation opposite of that in pOX38-*traP474*	This work
R100-1	Tra⁺ Cmʳ Faʳ Smʳ Spʳ Suʳ Tcʳ	14
λTn*phoA*	*b221 cI857 Pam3* with Tn*phoA* in or near *rex*	C. Manoil

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F        VVFQDGFQLKTVEEMALERTQSRAEEDNPESPVPVPPSAESHLNGFNTDQ        450

COLB2    VVVQDGFQLKTVEEMALERTQSRAEEDNPESPVPVPPSAESHLNGFNTDQ        450

R100-1   VVVQDGFQLKTVEEMALEQSQSTAEEGNPESPVPNPPSAESHLNGFNTDQ        450


F        MLKQLGNLNPQQFMSGSQGGGNDGK*                               475

COLB2    MLKQLGNLNPQQFMSGSQGGGNDGK*                               475

R100-1   MLKQLGNLNPQQFMSGAQEPAGRGCRQGGRKWRVMH*                    486
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FIG. 1. Alignment of amino acid sequences at the C termini of the TraB proteins of F, ColB2, and R100-1 plasmids. The 75 C-terminal residues of F and ColB2 and the 86 C-terminal residues of R100-1 are shown. The standard International Union of Pure and Applied Chemistry one-letter codes for the amino acids are used. Differences in residues at a given position are indicated in boldface type. The asterisks indicate stop codons.

by restriction mapping. The resulting plasmids were named pOX38-*traP470* (reverse orientation) and pOX38-*traP474* (forward orientation with respect to the polyomavirus *traY* promoter).

Nucleotide sequence accession numbers. The GenBank nucleotide sequence accession numbers are U51859 for R100-1 *traL-traP* and U51860 for ColB2 *traL-traP*.

RESULTS

Sequence analysis of the TraL, TraE, TraK, TraB, and TraP proteins. Portions of the *tra* regions of the F-like plasmids ColB2 and R100-1, containing the *traL*, -*E*, -*K*, -*B*, and -*P* genes (F coordinates, 2629 to 6250 [7]), were sequenced and compared with the equivalent region of F and with one another. Point mutations blocking the synthesis of either the *traE*, *traK*, or *traB* gene product completely abolish piliation and DNA transfer (1) and render cells carrying such plasmids resistant to pilus-specific phages. Mutations in *traL* also block pilus synthesis and transfer (unpublished results), while mutations in the *traP* gene, although not completely abolishing pilus synthesis, reduce the efficiency of DNA transfer and sensitivity to phage infection (see below).

TraL and TraE. The *traL* gene, which is immediately downstream of the pilin gene *traA*, encodes a 91-amino-acid (aa) polypeptide which is predicted to be a peripheral membrane protein with a domain for membrane association from aa 31 to 61 located in the cytoplasm. TraL has never been visualized in experiments to identify the various *tra* gene products, suggesting that its expression is highly regulated, possibly because of the large amount of secondary structure predicted to form within the *traL* coding sequence (for a review, see reference 7). The TraL gene protein has homology to TrbD in RP4 and to VirB3 in the Ti plasmid of *Agrobacterium tumefaciens* (among others [13]). The ColB2 and F TraL proteins are identical, while the R100-1 TraL protein is identical to that in F except for an isoleucine-to-valine transition at aa 38.

The *traE* gene product is a 188-aa-residue inner membrane protein which is predominantly hydrophilic in character and has a single predicted transmembrane domain (aa 12 to 33) near its N terminus, suggesting that it extends into the periplasmic space (for a review, see reference 7). It has previously been determined to be an inner membrane protein, which is in accordance with this prediction. The sequence of R100-1 *traE* revealed a number of single amino acid substitutions throughout the central portion of the molecule, including the following changes from the F *traE* sequence: T-47→P, A-53→G, A-56→V, S-86→P, T-88→I, R-118→L, N-121→K, A-140→V, V-147→I, L-148→R, D-154→N, and K-156→E. In addition, the R100-1 *traE* gene is interrupted by an IS*2* element at nucleotide 117. The TraE proteins of F and ColB2 are identical. The F TraE protein shares some sequence similarity with TrbF of RP4 and with VirB5 of the Ti plasmid (13).

TraK. The product of the *traK* gene is predicted to be a 242-aa periplasmic protein which is processed to a soluble product of 23.3 kDa (221 aa) (19). The *traK* gene product has been visualized on sodium dodecyl sulfate (SDS)-polyacrylamide gels as a 24-kDa protein by [35S]methionine labelling of λ transducing phages carrying the *traB-traC* region (18). Sequence differences between the F and ColB2 TraK proteins occur at three positions: M-33→I, L-50→M, and E-109→K. Three amino acid substitutions also occur in TraK of R100-1, namely, S-12→G, A-179→S, and A-192→V.

TraB. The product of the F *traB* gene contains 475 aa and is predicted to be a 50.5-kDa, predominantly hydrophilic protein exposed to the periplasm and anchored in the inner membrane via a short transmembrane segment from aa 13 to 33 (7). It has been visualized on SDS-polyacrylamide gels as a 60-kDa protein (18). Amino acid sequence comparisons reveal that the TraB protein of ColB2 is also 475 aa in length and has 100% sequence identity to F TraB while the R100-1 TraB contains 486 aa, with an additional 11 aa at the carboxy terminus (Fig. 1). Apart from the sequence changes shown in Fig. 1, an additional serine-to-proline substitution occurs in the R100-1 sequence at aa 141 (data not shown). The most notable feature of the DNA sequence of R100-1 *traB* is the sequence overlap between the end of *traB* and the beginning of *traP*, suggesting that translation of these proteins is coupled and that they act together to promote pilus biogenesis. While the F and ColB2 *traB* and *traP* sequences overlap by three codons, the R100-1 *traB* sequence extends 11 codons into the *traP* gene. One interesting aspect of the TraB sequence is the high proportion of proline (8%) and glycine (11.7%) residues, suggesting an extended structure for this protein through the periplasmic space. This protein has sequence similarity to the TrbI and VirB10 proteins of RP4 and the Ti plasmids, respectively (13).

TraP. A comparison of the *traP* sequences revealed short defined regions of variation in the TraP proteins of the three plasmids. The F TraP protein contains 196 aa residues, whereas the ColB2 and R100-1 proteins are 192 and 195 residues in length, respectively (Fig. 2). By using transducing phages carrying the *traP* gene, a 21.5-kDa polypeptide has been identified as the gene product of *traP* (18). The N-terminal residues of R100-1 TraP are significantly different from those of either the F or ColB2 proteins, while the major changes in ColB2 TraP occur in a stretch of amino acids at positions 81 to 96, with several single substitutions occurring at intervals throughout the distal end of the protein (Fig. 2). Secondary-structure predictions and hydropathy analysis reveal that TraP is a 20-kDa integral membrane protein with two transmembrane segments (aa 25 to 49 and aa 118 to 141, shown as overlined segments in Fig. 2). The altered N-terminal sequence in R100-1 TraP was predicted to be in the cytoplasm, while the loop containing the altered sequence in ColB2 was predicted to be in the periplasm (see below).

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       F      MANNMSSRQACHAARYVVARVLRGLFWCLKYTVILPLATMALMALFVLWK  50
       ColB2  MANNMSSRQACHAARYVVARVLRGLFWCLKYTVILPLATMALMALFVLWK  50
       R100-1 MPARRAEMAG-DALTFRVAFVVRWLLWVVRFVVIWPLATMALMALFVLWK  49


                   ▽▽                       ▽
       F      DNTTPGKLLVKEINFVRQTAPAGQFPVSECWFSSSDSSGRSEIQGICHYR 100
       ColB2  DNTTPGKLLVRKINFVRQTAPAGQFPVGECLPSLHDLPGAEE--KICRYR  98
       R100-1 DNTTPGKLLVKEINFVRQTAPAGQFPVSECWFSSSDSSGRSEIQDICHYR  99


                                        ▽▽ ▽
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       F      AADAADYVRETHRSLMQLVTALWATLALMYVSLAAITGKYPVRPGKMKCI   150
       COLB2  AADAADYVRETDRSLMQLVTAFWATLALMYMSFAAGTGKYPVGPGKMKCV   148
       R100-1 AADAADYVRETDRSLMQLVTAFWATLALMYMSFAAGTGKYPVLPGKMKCI   149



       F      RVVTADEHLKEVYTEDASLPGKIRKCPVYLPDDRTNRNNGDKNEHA*      196
       ColB2  RVATADERLKEVLAEDTSLPGKIRECHVYFPDDRTIRNNGE-NEHA*      193
       R100-1 RVVTADEYLKDVYTEDASLPGKIRKCHVYFPDDRTNRSNGDKNEHA*      195
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FIG. 2. Amino acid sequence alignment of the TraP proteins of F, ColB2, and R100-1 plasmids. The standard International Union of Pure and Applied Chemistry one-letter codes for the amino acids are used. Sequence differences are indicated in boldface type. Dashes indicate gaps introduced for alignment, and asterisks represent the stop codons. Overlined segments are the predicted transmembrane regions. The triangles mark the precise locations of Tn*phoA* insertions (refer to Fig. 3).

Tn*phoA* mutagenesis of F TraP. The orientation of TraP in the inner membrane was investigated by Tn*phoA* insertion mutagenesis with the λTn*phoA* delivery system described by Manoil (16). The plasmid pKI250, which contains the *traP-traV* genes (*traP*, *trbD*, *trbG*, *traV*, and *traR*; Table 2), was used since it could be stably maintained and it expressed the *tra* proteins at levels adequate for detection of kanamycin resistance and AP activity. Attempts to isolate *traP* alone on a separate plasmid resulted in plasmid instability. Since plasmids pKI250 and pKAF2, which contains the *traK-traP* genes (Table 2), can be stably maintained, expression of *traP* may require the sequences located immediately upstream or downstream. Transformants carrying transpositions on pKI250 were selected by hyperselection on medium containing kanamycin and BCIP. Colonies that were dark blue, indicating the presence of active alkaline phosphatase in the periplasmic space, were selected, and their DNAs were characterized by restriction mapping and sequencing. The transpositions giving rise to active AP were found to be located in *traP*, in *traV* (which codes for a lipoprotein in the outer membrane which faces the periplasm [4]), or in the β-lactamase gene of the vector. Insertions in the *traR* gene resulted in white colonies, confirming that TraR is a cytoplasmic protein (15). A schematic diagram of the predicted orientation of TraP in the inner membrane together with the sites of Tn*phoA* insertion is presented in Fig. 3. Several transpositions in TraP resulting in blue colonies (K-61, E-62, and S-78) were clustered in the predicted periplasmic domain which separated the membrane-spanning segments, while selected pale-blue colonies resulted from insertions within the second transmembrane region (L-128, Y-130, and S-132). Since no positive (blue) Tn*phoA* insertions were found near the N or C terminus of TraP, on the basis of the Tn*phoA* analysis and computer predictions, this protein is most probably oriented with its N and C termini in the cytoplasm, and a periplasmic loop, bracketed by transmembrane segments, is exposed in the periplasm (Fig. 3).

Construction and phenotypic characterization of pOX38 *traP* mutants. In order to determine the effects of a *traP* mutation on transfer efficiency and sensitivity to pilus-specific phages, a kanamycin resistance gene cassette (*kan*) was inserted at the unique *Pst*I site in *traP* in the plasmid pKI468
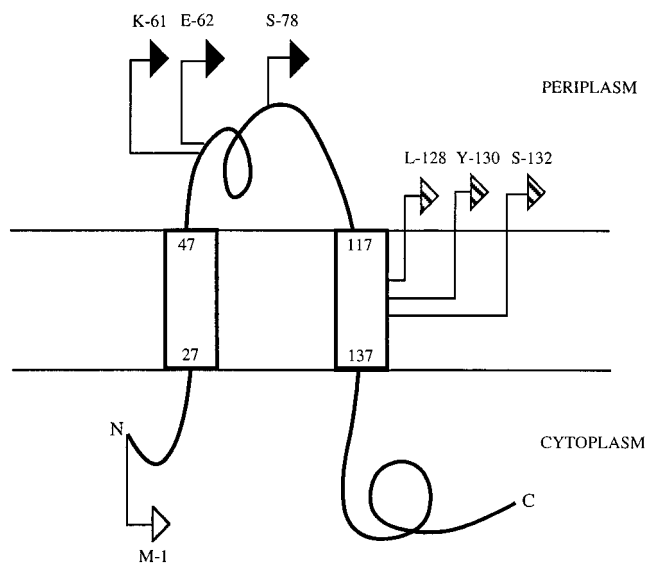

FIG. 3. Predicted orientation of the TraP protein in the inner membrane. Arrows mark the sites of Tn*phoA* insertions. Black arrowheads indicate high AP activity, hatched arrowheads represent low AP activity, and unshaded arrowheads indicate absence of AP activity. The predicted transmembrane segments are shown as vertical boxes. The letters and numbers refer to the amino acids and their positions in the protein, respectively. N and C are the amino and carboxy termini, respectively. Secondary-structure prediction was done by the method of Garnier et al. (10).
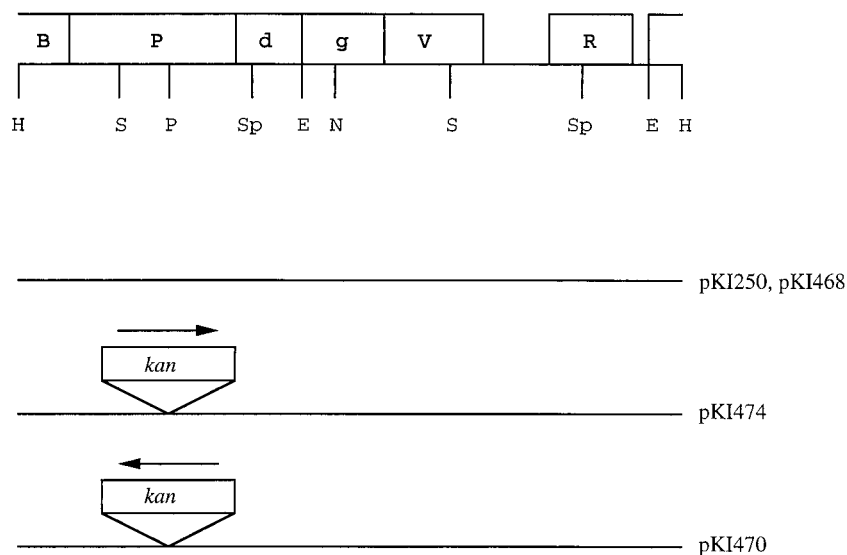
FIG. 4. Construction of *traP* mutations. The 2.5-kb *Hinc*II fragment of pKI250 is depicted. Boxes indicate the positions of the respective gene sequences; partial gene sequences are shown as open-ended boxes. Transcription of the *tra* and *trb* sequences is from left to right. The lines below the map show the *tra* region sequences carried by the various clones. The boxes and arrows in pKI474 and pKI470 mark the positions and directions of transcription of the *kan* cassette, respectively. Abbreviations: H, *Hinc*II; S, *Sma*I; P, *Pst*I; Sp, *Sph*I; E, *Eco*RI; N, *Nde*I.

(Fig. 4). This plasmid carries a 2.55-kb *Hinc*II fragment from the distal end of F *traB* to the beginning of *traC* (7). The resulting chimeric plasmids carried the *kan* insert oriented either in the same direction as *traP* (pKI474) or in the opposite direction (pKI470) (Fig. 4). These mutations were crossed onto the self-transmissible F plasmid derivative pOX38 to obtain pOX38-*traP474* and pOX38-*traP470*, respectively. The presence of these mutations was confirmed by restriction analysis.

The pOX38-*traP470* and pOX38-*traP474* plasmids were initially assayed for their ability to transfer and to support f1 phage growth (Table 3). The pOX38-*traP474* derivative had 4.4% of the mating efficiency of the wild-type pOX38-Km (3); however, when *traP* was supplied in *trans*, on pKI250, the efficiency of transfer increased to 26% of that of the wild type.

The results of a qualitative f1 phage spot test revealed a pattern similar to that of the mating efficiency assay, with pOX38-*traP474* having 0.1% of pOX38-Km's phage sensitivity; this was restored to 10% when pKI250 was present in *trans*, with the plaques having an unusually turbid appearance. The pOX38-*traP470* derivative, with the *kan* insert in the anti-*tra* orientation, exhibited dramatically reduced transfer levels and was resistant to f1 infection. The phenotypic differences between pOX38-*traP474* and -*traP470* might stem from a polarity effect of the *kan* insert on downstream genes, or it may be due to interference from an anti-*tra* RNA transcript originating from the *kan* promoter in pOX38-*traP470* (15).

The ability of *traP* from R100-1 and ColB2 plasmids to complement the pOX38-*traP474* mutation was analyzed by using chimeric plasmids pKAR2 and pKAB2, which express R100-1 *traP* and ColB2 *traP*, respectively. The mating results shown in Table 3 illustrate that the R100-1 *traP* complemented pOX38-*traP474* at levels comparable to those of its F counterpart, pKAF2. The ColB2 *traP* in pKAB2, however, complemented to 50% of the levels of pKAF2 and pKAR2. This reduction can be attributed to the lower expression of *traP* in pKAB2, which is not driven by the *lac* promoter as is the case in pKAF2 and pKAR2. Similarly, the efficiencies of plating of f1 for pOX38-*traP474* in the presence of all three types of *traP* genes were equivalent, suggesting that the *traP* gene is not responsible for attachment of the f1 phage. This result was further confirmed by showing that cells carrying pOX38-*traP474* supported f1 phage growth almost as well (less than 1 log reduction) as those carrying wild-type pOX38-Km. When cells were incubated with f1 phage at a multiplicity of infection of 1 on ice for 30 min, only 10-fold fewer phage were associated with the cells after centrifugation in pOX38-*traP474* than were associated with cells after centrifugation in pOX38-Km, suggesting that f1 attachment was not affected by the *traP474* mutation.

Immunoblot analysis of cells carrying pOX38-*traP474* and pOX38-*traP470* showed that these mutations did not affect the expression of pilin, which could be detected in the membrane

TABLE 3. Phenotypes of pOX38-*traP* mutants

Plasmid[a]	No. of transconjugants (per 100 donors)[b]	% of wild-type mating efficiency	f1 phage sensitivity[c]
pOX38-Km[d]	129	100	+++
pOX38-*traP470*	4.5×10^{-3}	2×10^{-5}	−
pOX38-*traP470*/pKI250	23	23	−
pOX38-*traP474*	4.36	2.4	+[e]
pOX38-*traP474*/pKI250	33	18	++[e]
pOX38-Km[d,f]	90	100	+++
pOX38-*traP474*/pKAF2	32	35.5	+++
pOX38-*traP474*/pKAR2	29	32	+++
pOX38-*traP474*/pKAB2	14.7	16.3	+++

[a] In the host *E. coli* XK1200.
[b] Transconjugants were selected on medium containing Km and Sm when JC3051 was used as the recipient.
[c] Phage sensitivity was determined by using a qualitative spot test. +++, 100% sensitive; ++, 10% of wild-type sensitivity; +, 0.1% of wild-type sensitivity; −, resistant.
[d] Wild-type control.
[e] Plaques were turbid.
[f] The recipient for this mating was *E. coli* MC4100.

TABLE 4. Effect of addition of 0.01 M sodium cyanide on the number of pili on cells carrying pOX38::Km or pOX38-*traP474*

E. coli XK1200 carrying plasmid:	No. of pili/no. of cells		% of cells with pili	
	− CN	+ CN	− CN	+ CN
pOX38-Km	12/47	14/126	25.5	11.1
pOX38-*traP474*	26/56	7/59	46.4	11.9
pOX38-*traP474* + pKI250	29/36	9/62	80.6	14.5

by using monoclonal antibodies specific for F pilin (data not shown; for a description, see reference, 15 and 17). Electron microscopic examination of cells carrying the two mutant pOX38 plasmids revealed that pOX38-*traP470* did not produce detectable numbers of pili while pOX38-*traP474* produced normal numbers of these structures (1.02 pili per cell, with 50% of 50 cells expressing pili) and retained the ability to bind the F-specific phage R17. The ability of cells carrying pOX38-*traP474* to undergo pilus retraction was assayed by counting the number of pili attached to cells in the presence and in the absence of 0.01 M sodium cyanide as described by Frost et al. (6) (Table 4). The presence of the *traP474* mutation was found to have no effect on the ability of these cells to undergo pilus retraction.

Is TraP directly involved in recipient cell recognition? Since pOX38-*traP474* had the phenotype expected of a mutant deficient in an adhesin for recipient cell recognition (normal piliation but poor mating efficiency), the role of TraP in recipient cell recognition was determined. We took advantage of the ability of pOX38-*traP474* to mate at reasonable levels to ascertain whether mutations in the recipient cell affected its mating efficiency. Since the F plasmid requires the PPEA moiety on the heptose I residue in the LPS of recipient cells in order to transfer DNA (2), the PPEA moiety, which is added to the LPS core structure by the product of the *rfaP* locus (22), may be the receptor recognized by the F transfer system prior to DNA transfer.

E. coli XK1200 donor cells carrying either wild-type pOX38-Km or the *traP* mutant pOX38-*traP474* were mated with recipients carrying mutations in the *rfa* loci. Recipients *E. coli* CS2057 (Δ*rfaGI1*) and CS2058 (Δ*rfaQJ2*) carry deletions extending from *rfaG* to *rfaI* and *rfaQ* to *rfaJ*, respectively, in addition to the *cps-5*::Tn10 mutation, which prevents the synthesis of capsular polysaccharide that is thought to block conjugation (2). Strains CS1977 and CS1981 are λ lysogens of CS2057 that carry the *rfaQJ* and *rfaQJP* genes, respectively, on the prophage. Matings with these strains were carried out at 30°C to prevent the induction of lysis. Since the efficiency of DNA transfer is reduced by 10-fold at 30°C (9), a wild-type control was also included at 30°C to compensate for the reduction (Table 5). The plasmid pOX38-Km showed 53 and 70% reductions in mating efficiency with CS2057 and CS2058, respectively, compared with the CS180-2 wild-type control, while pOX38-*traP474* showed a 70% reduction with both CS2057 and CS2058 (Table 5). When matings were performed with CS1977 and CS1981, the mating efficiency for CS1981 (*rfaP* present) was clearly higher than for CS1977 for both pOX38-Km and pOX38-*traP474*, demonstrating that both plasmids maintained a requirement for *rfaP* activity in the recipient cell. These results suggest that TraP is not responsible for the recognition of the PPEA group in the LPS of recipient cells and is probably neither at the pilus tip nor exposed on the cell surface. It is possible that TraP may play a minor role in the pilus biogenesis pathway, perhaps as an

TABLE 5. Mating efficiencies of *E. coli*/pOX38-Km and *E. coli*/ pOX38-*traP474* with recipients carrying mutations in *rfa* loci

Donor strain *E. coli* XK1200 carrying plasmid:	*E. coli* recipient (relevant phenotype, selection temp)	Trans-conjugants/ 100 donors[a]	% Mating efficiency[b]
pOX38-Km	CS180-2 (37°C)	100	100
	CS2057 (Δ*rfaGI1*, 37°C)	4.7	4.7
	CS2058 (Δ*rfaQJ2*, 37°C)	3.0	3.0
pOX38-*traP474*	CS180-2 (37°C)	0.7	100
	CS2057 (Δ*rfaGI1*, 37°C)	0.02	2.9
	CS2058 (Δ*rfaQJ2*, 37°C)	0.02	2.9
pOX38-Km	CS180-2 (30°C)	70	100
	CS1977 (*rfaQ⁺G⁺*, 30°C)	1.05	1.5
	CS1981 (*rfaQ⁺G⁺P⁺*, 30°C)	12	17
pOX38-*traP474*	CS180-2 (30°C)	0.440	100
	CS1977 (*rfaQ⁺G⁺*, 30°C)	0.005	1.1
	CS1981 (*rfaQ⁺G⁺P⁺*, 30°C)	0.124	28

[a] Mating efficiencies are expressed as the number of transconjugants per 100 donor cells.
[b] Percent mating efficiency is expressed as the percentage of the mating efficiency versus the CS180-2 wild-type control.

accessory element of the transmembrane complex involved in pilin polymerization.

DISCUSSION

The mosaicism found in the *tra* genes of closely related plasmids in the IncF group can reflect specificity in each transfer system, as demonstrated by the sequence differences of proteins required for DNA transfer (TraM and TraY) which cannot substitute for each other (25). Sequence differences in genes from closely related plasmids (R100-1 and ColB2) which complement F mutations could reflect small variations in the mechanism of mating pair formation and pilus biogenesis among these plasmids. The more subtle differences in sequence in proteins involved in pilus synthesis and retraction, and mating pair formation may provide clues to the functions of and protein-protein interactions among the many *tra* proteins involved in these processes.

Although mutational analyses have shown that the *traE*, *traK*, and *traB* genes are essential for piliation and transfer (1), very little is known about their specific roles in pilus synthesis. TraB is predicted to be a hydrophilic protein that extends into the periplasmic space and is anchored in the inner membrane via a single transmembrane segment near the N terminus. The high concentrations of glycine and proline in TraB are characteristic of proteins with an extended conformation, such as VirB10 and TonB (5). Recently, the TraB homolog VirB10 (of the Ti plasmid) was shown to be tightly associated with the membrane fraction of *A. tumefaciens*, possibly in a complex spanning the cell envelope (5) which is similar to the complex proposed for the conjugative apparatus (7).

TraP is predicted to have a single periplasmic domain bracketed by two transmembrane segments, with its N and C termini in the cytoplasm. Thus, the changes in sequence in TraB of R100-1 might occur in the periplasm while the change at the N terminus of R100-1 TraP might be in the cytoplasm. This would suggest that the sequence changes in these two proteins do not define interactions with each other; rather, each of these proteins may in turn interact with other *tra* proteins. The coupling of the stop and start codons of *traK* and *traB* as well as the overlap between *traB* and *traP* and

between *traP* and *trbD* supports the concept of coordination of function among these gene products. Interestingly, the *traKBP* genes could be expressed from *lac* promoters of multicopy vectors such as pUC18, but attempts to clone *traP* away from *traKB* were unsuccessful. Also, the cloning of PCR products of regions of R100-1 and ColB2 containing *traP* alone were unsuccessful, and larger PCR products incorporating *traKBP* were required. This was also true for pKI250, since subcloning of *traP* away from the *trbD-traR* genes resulted in plasmid instability. This suggests that the *traK-trbD* gene products (at a minimum) act in a coordinated manner during pilus biogenesis.

Mutations in *traP* had a small effect on plasmid transfer efficiency and a minimal effect on pilus synthesis and function, suggesting that it is not required for F transfer in *E. coli*. The notion that TraP might be the adhesin at the pilus tip was negated by the inability of the R100-1 and ColB2 *traP* genes to change the mating phenotype of the F plasmid or to affect f1 phage sensitivity. The complementation of pOX38-*traP474* with pKAR2 was complicated by the presence of the *traKB* genes in addition to the *traP* gene of R100-1. Thus, the complete complementation of the pOX38-*traP474* mutation by R100-1 *traP* could have involved the R100-1 *traKB* gene products. Experiments to create chimeric conjugative plasmids containing only one copy of each gene are currently in progress.

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