F plasmid, an autoregulatory homotetramer, is essential for F plasmid bacterial conjugative transfer, one of the major mechanisms for horizontal gene dissemination. TraM cooperatively binds to three sites (sbmA, -B, and -C) near the origin of transfer in the F plasmid. To examine whether or not tetramerization of TraM is required for autoregulation and F conjugation, we used a two-plasmid system to screen for autoregulation-defective traM mutants generated by random PCR mutagenesis. A total of 72 missense mutations in TraM affecting autoregulation were selected, all of which also resulted in a loss of TraM function during F conjugation. Mutational analysis of TraM defined three regions important for F conjugation, including residues 3–10 (region I), 31–53 (region II), and 80–121 (region III); in addition, residues 3–47 were also important for the immunoreactivity of TraM. Biochemical analysis of mutant proteins indicated that region I defined a DNA binding domain that was not involved in tetramerization, whereas regions II and III were important for both tetramerization and efficient DNA binding. Mutations in region III affected the cooperativity of binding of TraM to sbmA, -B, and -C. Our results suggest that tetramerization is important for specific DNA binding, which, in turn, is essential for traM autoregulation and F conjugation. These findings support the hypothesis that TraM functions as a “signaling” factor that triggers DNA transport during F conjugation.

Bacterial conjugation is a major mechanism for horizontal gene transfer and is defined as the unidirectional transfer of single-stranded DNA from a donor to a recipient cell. This process occurs in response to an uncharacterized mating signal generated by mating pair formation initiated by intimate cell-to-cell contact (1, 2). Conjugation requires an origin of transfer (oriT) in cis to the transferred DNA that contains a nic site as well as multiple binding sites for DNA-binding proteins that together form the relaxosome. Cleavage and re-ligation at the nic site by TraI, the relaxase, is in equilibrium until a stable mating pair has formed, which triggers DNA unwinding and transport between cells (2, 3).

The F plasmid is the paradigm for a large group of conjugal plasmids in the IncF incompatibility complex that carries genes important for human and veterinary medicine, such as antibiotic resistance and toxin production (4). The genes responsible for F conjugation are located in the 33-kb F major transfer (tra) region with traM and trac upstream of the major tra operon (traY and traI) (5). F plasmid TraM, which is essential for F conjugation, is not required for pilus synthesis, mating pair formation, or nicking at oriT in vivo (1, 6–8). Consequently, TraM is thought to signal that donor and recipient cell contact has been made, thereby activating DNA transfer (1).

TraM (127 aa, 14.5 kDa) is a cytoplasmic protein that cooperatively binds to three sites (sbmA, -B, and -C) near oriT in the F plasmid (9, 10). The sites with the highest affinities (sbmA and -B, respectively; $K_a = 2 \times 10^8 \text{ M}^{-1}$) overlap the two traM promoters (collectively called P$_{traM}$), allowing autoregulation of traM transcription (9–11). The lowest affinity site, sbmc (the promoter for sbMC, 70-aa-long, 0.7 $\times 10^8 \text{ M}^{-1}$), is located nearest to oriT (10). Deletion of sbmA and sbmc from an oriT fragment cloned into a multicopy plasmid decreases conjugative transfer by 100-fold, whereas removal of sbmc decreases transfer by an additional 100-fold (12).

TraM forms tetramers in solution; the oligomerization equilibrium between tetramers and monomers can be described as two-state, suggesting that other oligomerization intermediates are not detectable (13). This is consistent with evidence that R1 TraM binds to DNA as a tetramer (14) but argues against a model in which TraM initially binds to DNA as a dimer (10). Deletion analysis has indicated that a TraM fragment of aa residues 2–55 forms dimers, whereas a TraM fragment consisting of the C-terminal 70 aa (58–127) residues forms tetramers (13), suggesting that both regions are involved in TraM oligomerization.

The N-terminal region of TraM is important for specific DNA binding and is required for traM autoregulation and F conjugation (13, 15); however, the role of the C-terminal region remains ambiguous. The importance of TraM tetramerization for autoregulation and F conjugation is unclear; similarly, the structural domains of TraM responsible for the specificity and cooperativity of DNA binding are unknown. A preliminary mutational analysis of TraM has shown that a missense mutation in either the C- or N-terminal regions can affect TraM in both autoregulation and F conjugation (16). In this work, 76 point mutations in $traM$ were characterized for their effect on autoregulation and F conjugation; selected mutants were also characterized for their immunoreactivity, oligomerization, and DNA binding specificity. The results suggest that oligomerization is crucial for TraM function in DNA binding that, in turn, is essential for autoregulation and F conjugation.

* This work was supported in part by the Canadian Institutes for Health Research. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ Supported by an Alberta Ingenuity Fund Studentship.

§ To whom correspondence should be addressed: Dept. of Biological Sciences, CW405 Biological Sciences Bldg., University of Alberta, Edmonton, Alberta T6G 2E9, Canada. Tel.: 780-492-0672; Fax: 780-492-9234; E-mail: laura.frost@ualberta.ca.

† The abbreviations used are: aa, amino acid(s); P$_{traM}$, the traM promoters; oriT, origin of transfer; X-gal, 5-bromo-4-chloro-3-indolyl-$\beta$-D-galactoside; SEC, size exclusion chromatography; BSA, bovine serum albumin.
Experimental Procedures

Growth Media and Bacterial Strains—Cells were grown in LB (Luria-Bertani) broth or on LB solid medium containing appropriate antibiotics or other supplements (17). Antibiotics were used at the following final concentrations: ampicillin, 50 μg/ml; kanamycin, 25 μg/ml; spectinomycin, 100 μg/ml; nalidixic acid, 40 μg/ml; chloramphenicol, 50 μg/ml. X-gal was used at a final concentration of 100 μg/ml. Isopropyl-thio-β-D-galactoside was used at a final concentration of 1 mM. The following Escherichia coli strains were used: XK1200 (F’ ΔlacU124 ΔndonA gal attA bio) gvrA (Nalr) (18), ED24 (F’ Lac’ Spe’ (19), DH5α (lacU169 (880 lacZΔM15) supE44 hsdR17 recA1 endA1 gyrA96 relA1 thi-1 rilA1), and BL21-DE3 (F’ dcm ompT hsdS (rB- mB-) gal λ(DE3), Stragatene).

DNA Manipulation, PCR, DNA Sequencing, and Sequence Analysis—DNA purification, manipulation, and PCR reactions followed standard procedures (17) or protocols from the manufacturers. Vent DNA polymerase (New England Biolabs) was used for all PCR reactions except for random PCR mutagenesis that used Taq DNA polymerase. Miniprep and Gel Extraction kits (Qiagen) were used for plasmid purification, manipulation and PCR reactions followed standard procedures (20), and BL21-DE3 (F’ dcm ompT hsdS (rB- mB-) gal λ(DE3), Stragatene).

TABLE I

| Bacterial plasmids and oligonucleotides | Description and references |
|----------------------------------------|-----------------------------|
| pACPM242fs:lacZ                         | pACYC184 with a P<sub>pol</sub>-lacZ fusion (16) |
| pBluescript KS+                         | A high copy number cloning vector (34) |
| pJLM102                                | pTT7-4 with a DraI-BglII fragment containing F plasmid P<sub>pol</sub> and traM (this work) |
| pJLM103                                | pJLM102 derivative with a traM mutation, R31E (this work) |
| pJLM104                                | pJLM102 derivative with a traM mutation, 1109T (this work) |
| pJLM400                                | pBluescript KS+ with traM expressed from the lac promoter (this work) |
| pLDLF007                               | pTT7-4 with a DraI-DraI fragment containing F plasmid P<sub>pol</sub> and traM (9) |
| pOX38-K                                | The F transfer region and RepFIA fused to a kanamycin resistance gene (35) |
| pOX38-MK                               | pOX38 with traM disrupted by a kanamycin resistance gene (11) |
| pRF911                                 | pBR222-derived pBlnd2 with shmA (10) |
| pRFM200                                | pRFM200 derivative with a deletion containing most of traM (16) |
| pRFM200-Mdel                            | pRFM200 derivative with a deletion containing most of traM (16) |
| pTT7-4 and pTT7-5                       | Medium-copy cloning vectors (36) |
| pJLU3                                  | 5’-CTATAGGGAGACCGGAATTCG-3’ |
| pJLU4                                  | 5’-CGATAAGTTGGTGCGTCGGAG3’ |
| pJLU80                                 | 5’-TAGGGTATACAGAGGCCC-3’ |
| pJLU81                                 | 5’-GGTTGGCTGACGGCCTAGGC-3’ |

* The nucleotide sequences of the F plasmid and pBR322 are available under GenBank™ accession numbers NC_002483 and J01749, respectively.

Oligomerization and DNA Binding of F Plasmid TraM

| Plasmid and oligonucleotide | Description and references |
|----------------------------|-----------------------------|
| pRFM200 derivatives and pJLM400 derivatives, which contain different traM mutants, are not listed here. They are named after the corresponding mutations (Table II). | |

Abbreviations: D-galactoside was added to the culture to a final concentration of 1 mM, and the culture was grown for another 2 h before harvesting. Approximately 150 A<sub>600</sub> cells of each strain were used for purification of each protein. The cell pellet was suspended in 6 ml of B-Per® bacterial protein extraction reagent (Pierce) with one tablet of Complete, Mini-protease inhibitor mixture (Roche Applied Science); the soluble fraction of the cells was extracted according to the manufacturer’s instructions. All the following steps were performed at 4 °C or on ice. Ammonium sulfate (720 mg) was dissolved in the extracted soluble fraction. After centrifugation at 27,000 × g for 10 min, the supernatant was transferred into a new centrifuge tube, in which 480 mg of ammonium sulfate was dissolved. After centrifugation at 27,000 × g for 10 min, the supernatant was carefully aspirated and discarded. The precipitate was dissolved in 2 ml of malonic acid (50 mM, pH 5.5), and the solution was desiccated previously (16). Briefly, potential traM mutants cloned in pRFM200 derivatives were transformed into DH5α cells containing pACP242fs:lacZ. The transformed cells were plated on solid LB medium containing X-gal, chloramphenicol, and ampicillin. Transformants were incubated for 24 h at 37 °C; dark blue colonies were collected for further characterization. Plasmid DNA from each dark blue colony was extracted and sequenced using primers pJLU3 and pJLU4 to locate mutations in traM.

β-Galactosidase Assays—A fresh, single colony was inoculated into LB broth containing appropriate antibiotics and grown at 37 °C with shaking for 16 h. A 200-μl sample was used for determining β-galactosidase activity as described by Miller (21) and reported as Miller units. The values were calculated using the equation: 1000 (A<sub>420</sub>/A<sub>660</sub>), where t = time of reaction (minutes) and v = volume of culture added (ml).

Donor Ability Assays—E. coli XK1200 and ED24 were used as donor and recipient strains, respectively. The mating experiments were performed as previously described (22). Donor ability was calculated as the number of transconjugants divided by the number of donors.

Blue Native PAGE and Immuno blot Analysis—0.1 A<sub>600</sub> (except where specified) of exponentially growing cells or a specified amount of pure protein were separated by a 15% SDS-polyacrylamide gel with a 7% stacking gel. TraM was assayed by Coomassie Blue staining (17) or by immunoblot as described by Penfold et al. (11). Immunoblot analysis was performed using rabbit anti-TraM antiserum (9) at a 1:10,000 dilution and donkey anti-rabbit secondary antibodies linked to horse radish peroxidase (Amersham Biosciences) at a 1:20,000 dilution.

Donor Ability Assays—E. coli XK1200 and ED24 were used as donor and recipient strains, respectively. The mating experiments were performed as previously described (22). Donor ability was calculated as the number of transconjugants divided by the number of donors.

Blue Native PAGE—Native gel samples were prepared as previously described (16). A 6 to 15% gradient polyacrylamide gel (pH 7.0) with a 4% stacking gel (pH 7.0) was used for electrophoresis following the procedures described by Schagger and von Jagow (23) and Schagger et al. (24). Proteins separated by blue native gels were transferred to polyvinylidene difluoride membranes for immunoblot analysis with anti-TraM antiserum.

Overexpression and Purification of TraM and Its Mutants—BL21-DE3 cells containing pRFM200 or its derivatives were grown in 250 ml of LB broth containing ampicillin at 37 °C with vigorous shaking. After 3 h, isopropylthio-β-D-galactoside was added to the culture to a final concentration of 1 mM, and the culture was grown for another 2 h before harvesting. Approximately 150 A<sub>600</sub> cells of each strain were used for purification of each protein. The cell pellet was suspended in 6 ml of B-Per® bacterial protein extraction reagent (Pierce) with one tablet of Complete, Mini-protease inhibitor mixture (Roche Applied Science); the soluble fraction of the cells was extracted according to the manufacturer’s instructions. All the following steps were performed at 4 °C or on ice. Ammonium sulfate (720 mg) was dissolved in the extracted soluble fraction. After centrifugation at 27,000 × g for 10 min, the supernatant was transferred into a new centrifuge tube, in which 480 mg of ammonium sulfate was dissolved. After centrifugation at 27,000 × g for 10 min, the supernatant was carefully aspirated and discarded. The precipitate was dissolved in 2 ml of malonic acid (50 mM, pH 5.5), and the solution was desiccated previously (16). Briefly, potential traM mutants cloned in pRFM200 derivatives were transformed into DH5α cells containing pACP242fs:lacZ. The transformed cells were plated on solid LB medium containing X-gal, chloramphenicol, and ampicillin. Transformants were incubated for 24 h at 37 °C; dark blue colonies were collected for further characterization. Plasmid DNA from each dark blue colony was extracted and sequenced using primers pJLU3 and pJLU4 to locate mutations in traM.

β-Galactosidase Assays—A fresh, single colony was inoculated into LB broth containing appropriate antibiotics and grown at 37 °C with shaking for 16 h. A 200-μl sample was used for determining β-galactosidase activity as described by Miller (21) and reported as Miller units. The values were calculated using the equation: 1000 (A<sub>420</sub>/A<sub>660</sub>), where t = time of reaction (minutes) and v = volume of culture added (ml).

Donor Ability Assays—E. coli XK1200 and ED24 were used as donor and recipient strains, respectively. The mating experiments were performed as previously described (22). Donor ability was calculated as the number of transconjugants divided by the number of donors.
centrifuged at 27,000 × g for 10 min. The supernatant was brought to 2.5 ml with malonic acid (50 mM, pH 5.5) and was desalted on a PD10 column (Amersham Biosciences). After passing through a 0.45-μm Millex® syringe-driven filter (Millipore), the desalted protein extract was loaded onto a cation-exchange column (Mono S HR 5/5, Amersham Biosciences) using an Amersham Biosciences fast protein liquid chromatography, model LCC-500. The column was eluted with malonic acid (50 mM, pH 5.5) and a 0 to 1 M NaCl gradient. Because TraM has very low UV absorbance, eluted fractions were examined by 15% SDS-polyacrylamide gels with Coomassie Blue staining, and protein peaks were further confirmed by immunoblotting with anti-TraM antiserum. Wild type TraM and the mutants V4A, F35S, A37V, Q53L, and F120L were eluted at 0.3–0.5 M NaCl. I109T, F120S, F121V, and F121S were eluted with SEC buffer (50 mM sodium phosphate, 150 mM NaCl, pH 7.2), and the eluate was collected in 2-ml fractions. Fractions from B9 (void volume) to E12 (one column volume) were examined. The major peak fractions of each protein were concentrated and desalted, and the buffer was exchanged for Tris-HCl (50 mM, pH 7.6) using an Amicon® ultracentrifuge filter (Millipore) to a final volume of 50 μl. Protein concentration was determined using BCA protein assays (Pierce) following the manufacturer’s instructions.

Analytical Size Exclusion Chromatography—Purified TraM or its mutants (5 μg) were brought to 1 ml with SEC buffer and loaded onto a Hiload® 16/60 Superdex 75 prep grade column, Amersham Biosciences). The column was eluted with SEC buffer (50 mM sodium phosphate, 150 mM NaCl, pH 7.2), and the eluate was collected in 2-ml fractions. Fractions from B9 (void volume) to E12 (one column volume) were examined. The major peak fractions of each protein were concentrated and desalted, and the buffer was exchanged for Tris-HCl (50 mM, pH 7.6) using an Amicon® ultracentrifuge filter (Millipore) to a final volume of 50 μl. Protein concentration was determined using BCA protein assays (Pierce) following the manufacturer’s instructions.

Electrophoretic Mobility Shift Assays—DNA fragments containing sbmA and sbmABC were amplified by PCR from pRF91 and pRF90, respectively, using primers JLU80 and JLU91. The resulting mixtures were concentrated in a Savant SpeedVac and separated by a 2% agarose gel. The sbmA and sbmABC fragments were isolated from the agarose gel and were quantified using an Ultraspec 3000 (Amersham Biosciences). Each binding reaction contained 40 nM of sbmA or sbmABC, 50 mM Tris-HCl (pH 7.6), 10% glycerol, 1 mM dithiothreitol, 300 μM bovine serum albumin, and 1.5 μg poly(dIdC) with a final volume of 15 μl. After addition of a specified amount of purified TraM or its mutant proteins, each reaction mixture was incubated at 30 °C for 20 min. The resulting mixture was added with 3 μl of 6× load dye (0.25% bromphenol blue, 30% glycerol) and loaded onto a 2% agarose gel that had been pre-run at 4 °C and 30 mA in TBE (90 mM Tris borate, 1 mM EDTA) for 30 min. The gel was run at 4 °C and 30 mA until the bromphenol blue dye reached the bottom of the gel. DNA was visualized by ethidium bromide staining.

RESULTS

Isolating Autoregulation-defective traM Mutants—A two-plasmid system was developed to select autoregulation-defective traM mutants generated by random PCR mutagenesis (16). In this system, pRFM200 expresses traM at a level comparable to that of the F plasmid. pACPM24fs::lacZ is a transcriptional fusion vector that carries the PtraM promoter fused to lacZ with a −1 frameshift mutation at the beginning of lacZ to drastically reduce (−500-fold) its activity and allow differentiation of regulated and unregulated lacZ expression. This results in a simple detection method for autoregulation-defective TraM mutants that give dark blue colonies rather than the pale blue colonies characteristic of wild-type TraM (see “Experimental Procedures”).

A total of 234 mutant colonies were selected from ~25,000 transformants. DNA sequencing analysis revealed 135 pRFM200 derivatives with point mutations in traM, whereas the remaining 99 colonies contained frameshift mutations, multiple point mutations, or mutations within the ribosome-binding site or start codon of traM, which were discarded. The 135 point mutants included 72 missense, 4 nonsense, and 59 redundant mutations that were located in 56 different amino acid residues of TraM (Fig. 1).

To assay the effect of the mutations on autoregulation, β-galactosidase (LacZ) activity assays were performed on cells containing pACPM24fs::lacZ and the mutant derivatives of pRFM200. Cells containing pRFM200 derivatives had LacZ activities ranging from 18 to 45 Miller units, whereas cells containing pRFM200 (wild type traM) or pRFM200-Mdel (a traM-deleted pRFM200 derivative) had activities of 8 Miller units or 42 Miller units, respectively (Table II). Except for nonsense mutations, the cells with LacZ activities of ~40 Miller units contained point mutations within the first 47 codons of traM.

Some N-terminal Mutations Reduced TraM Immunoreactivity—As previously shown (16), pRFM200 and its mutant derivatives expressed similar levels of TraM as determined by immunoblot analysis (Table II). Thus, loss of autoregulation of PtraM was not due to decreased levels of TraM. Some mutants with missense mutations within the first 47 codons of traM were difficult to detect by immunoblot using polyclonal anti-TraM antiserum, whereas S79*, one of the four nonsense mutants, was fully detectable (Table II (16)). To distinguish be-
between protein instability and reduced immunoreactivity with TraM antisem, wild-type traM and selected mutants were cloned into pBluescript KS+ to give pJLM400 and its mutant derivatives, respectively, which expressed TraM at levels sufficient for detection by Coomassie Blue staining of SDS-polyacrylamide gels (Fig. 2A). In the absence of glucose, the pJLM400 mutant derivatives expressed TraM at levels equivalent to pJLM400 (wild type TraM); however, immunoblot analysis of an equivalent gel revealed differing levels of immunoreactivity to the TraM antisem (Fig. 2B). Thus, mutations in the first 47 aa of TraM affected the immunoreactivity of TraM rather than reducing protein stability.

**Three Regions of TraM Are Important for F Conjugation**—To assess the effects of different mutations on the function of TraM in F conjugation, pRF200 or its mutant derivatives was transformed into E. coli X1K1200 containing the traM-deficient F plasmid pOX38-MK3. Mating efficacy assays showed that pRF200 (traM) restored pOX38-MK3 transfer to a frequency of 4 × 10^-1 transconjugants per donor (100%), whereas none of the pRF200 derivatives complemented pOX38-MK3 transfer completely (Table II). The most severe mutations were concentrated in three regions of TraM, I, II, and III, which correspond to residues 3–10, 31–53, and 80–121, respectively (Fig. 1).

Two mutants were selected to determine the relationship between TraM levels, Prma2 autoregulation and function during conjugation. A region II mutant, K31E, and a region III mutant, I109T, which were highly defective for F conjugation (Fig. 1 and Table II), were cloned downstream of P<sub>traM</sub> in a medium-copy vector, pT7-4 (Table III). Both pJLM103 (Prma2K31E) and pJLM104 (Prma2I109T) overexpressed TraM compared with wild-type pJLM102 without affecting the growth rate of the cells (data not shown). In cells containing pACPM24fl::lacZ, pJLM104 (I109T) repressed Prma2 almost as well as wild type TraM (pJLM102), whereas pJLM103 (K31E) did not. In contrast, overexpression of either I109T or K31E resulted in lower levels of complementation of pOX38-MK3 for transfer, suggesting that autoregulation and F conjugation are genetically distinct functions of TraM. When co-resident with pOX38-MK<sub>tra</sub>, a wild-type F plasmid derivative, both K31E and I109T decreased the transfer frequency of pOX38-MK<sub>tra</sub> by more than 400-fold (Table III), suggesting negative dominance of the mutants over wild-type TraM.

**Analysis of TraM Mutants Using Blue Native PAGE**—Auto-regulation could be affected by mutations that alter either DNA binding affinity or the oligomerization ability of TraM. The ability of TraM and its mutants to form tetramers was inves-
tigated using blue native gel electrophoresis (Fig. 3). During blue native gel electrophoresis, the proteins are bound with negatively charged Coomassie Blue G-250. This masks the effect of mutations on the intrinsic charge of a protein that would alter its electrophoretic mobility during conventional native gel electrophoresis (23). As expected, wild-type TraM migrated predominantly as a single band that was similar in size to BSA monomers (66 kDa), indicating that TraM formed tetramers (~58 kDa; Fig. 3A). Higher order species of TraM were also detected that are characteristic of the propensity of TraM to aggregate. Most of the mutants tested, especially C-terminal mutants, also exhibited species smaller than tetramers. These smaller species were not degradation products, because SDS-PAGE of equivalent samples revealed no degradation of TraM or its mutants (Fig. 3B). Due to the resolution limit of blue native gel electrophoresis (23, 24), these smaller species could be either dimers (~29 kDa) or monomers (~14.5 kDa).
Oligomerization and DNA Binding of F Plasmid TraM

Oligomerization of Purified TraM and Its Mutants—Twenty-one TraM mutants with point mutations in regions I, II, or III were selected for purification and further characterization. Wild type TraM and 13 mutants were successfully purified (see “Experimental Procedures”), whereas R24Q, K31E, L42P, L47P, S89F, S95P, F100S, and S114P were deemed to have significant changes in charge or conformation because of their inability to bind to the cation exchange column. During preparative size exclusion chromatography (SEC), TraM and the 13 mutants were present as a single major peak with one or two minor peaks. Two characteristic patterns for wild-type and mutant TraM, with F121S serving as an example, are shown in Fig. 4A. Small amounts of TraM and the mutant proteins were consistently found in the void volume fractions (B11), suggesting that TraM either aggregated or bound nonspecifically to protein contaminants that could be detected by Coomassie Blue staining (Fig. 4A).

The oligomerization status of TraM was further examined by

**Fig. 4. Preparation and analysis of TraM and its mutants using size exclusion chromatography.** Samples were analyzed by 15% SDS-polyacrylamide gels. Fraction numbers are marked above each lane. Peaks of different protein standards are shown above the figures. Blue dextran is an indicator of the column void volume (2000 kDa); BSA represents bovine serum albumin monomer (66 kDa); CEA stands for chicken egg albumin (45 kDa); CA stands for carbonic anhydrase (29 kDa). A, preparative SEC fractions of TraM and F121S as detected by Coomassie Blue staining. The first lane on the left of each figure was loaded with protein standards. B, analytical SEC fractions of TraM and F121S as detected by immunoblot with anti-TraM antiserum. C, analytical SEC fractions of purified N5D and A37V as detected by immunoblot with anti-TraM antiserum. D, analytical SEC fractions of purified S79* as detected by immunoblot with anti-TraM antiserum.
analytical size exclusion chromatography using protein in the major peak from preparative SEC (see “Experimental Procedures”). TraM was detected by immunoblot, because the molar extinction coefficient of TraM, which lacks tryptophan, is very low. TraM was resolved as a single peak at fraction C4 (Fig. 4B), which corresponded in size to a tetramer (\( \approx 58 \) kDa). Almost no material corresponding to the peak containing higher aggregates (B1) during preparative SEC of either TraM or F121S was detectable (Fig. 4B).

The elution profiles of the TraM mutants during analytical SEC could be divided into four groups. F121S, in the first group, had a major peak at C6 corresponding to 45 kDa and a minor one corresponding in size to a dimer (29 kDa; Fig. 4B). Because the shape of a protein can affect its mobility during SEC (29), the major peak for F121S was thought to contain tetramers with altered conformation. This first group also contained I109T, F120S, and F121V that shared identical elution profiles with F121S (Table IV). In the second group, N5D (Fig. 4C) as well as V4A, N10D, F35S, R48C, and F120L (Table IV) had elution profiles identical to wild-type TraM, indicating the presence of tetramers. In the third group, A37V (Fig. 4C) and Q53L (Table IV) had a major peak identical to that of TraM tetramers and a minor peak at C7, which was thought to be dimers that differed from those of F121S in conformation. In the fourth group, S79*, a truncated TraM fragment of 36 kDa tetramers (Fig. 4C), had a major peak at C6 corresponding to 45 kDa and a minor peak at C7 that was close to the expected position of 36-kDa tetramers (Fig. 4D). These results indicated that the smaller-than-tetramer species observed during blue native gel electrophoresis were probably dimers. Thus mutations that affected tetramerization correlated with their defects in autoregulation and F conjugation, suggesting that tetramerization is important for the TraM function.

Specific DNA Binding of TraM and Its Mutants—Electrophoretic mobility shift assays were performed to determine the ability of TraM and its mutants to bind DNA. They could be categorized into four groups according to their patterns of binding to DNA fragments containing sbmAABC, the three TraM binding sites. In group A (Fig. 5A), wild-type TraM bound to sbmAABC with high affinity. At 600 nM TraM (equal to 150 nM TraM tetramers), 40 nM sbmAABC was almost completely shifted to a position corresponding to binding to sbmA (10). A 3-fold increase of protein concentration resulted in a second shift to a position corresponding to binding to all three sites in sbmAABC (10). F120L bound to sbmAABC with an affinity slightly lower than wild-type but had the two shifted bands characteristic of the cooperative binding of TraM (Fig. 5A).

Group B proteins (N5D and S79*) did not shift sbmAABC significantly at the highest concentration of protein tested (6000 nM), indicating a loss of DNA binding ability for these two mutants (Fig. 5B). In group C, V4A, N10D, F35S, A37V, R48C, and Q53L required 3- to 10-fold more protein than wild-type TraM to completely shift sbmAABC, indicating decreased DNA binding affinity for these mutants (Fig. 5C and Table IV). In group D (Fig. 5D), I109T, F120S, F121V, and F121S, had lowered binding affinities for sbmAABC similar to group C mutants. They shifted sbmAABC into smeared bands, which could represent a mixture of binding complexes of different electrophoretic mobilities suggesting a loss of cooperativity during binding (Fig. 5D). The cooperativity of binding to DNA by the mutants I109T and F121V was further investigated by electrophoretic mobility shift assay using a DNA fragment containing only sbmA, the site with the highest affinity for TraM. Both mutants shifted sbmA to a single band rather than a smear, similar to wild-type TraM (Fig. 5E), although the affinity for the DNA was reduced in both cases. Thus, the smeared shifted bands, characteristic of group D mutants, appeared to be the result of a loss of cooperativity during DNA binding, with the mutant proteins binding all three sites in no particular order (10, 25). All three regions (I, II, and III; Fig. 1) appeared to be important for TraM binding, with region III defining the ability of TraM to bind to sbmA, -B, and -C in order, which is a feature of cooperativity.

**DISCUSSION**

The analysis of point mutations in TraM revealed that distinct regions of the protein were involved in defining reactivity with anti-TraM antiserum as well as the properties of autoregulation and competence for DNA transfer. The first 47 aa of TraM appeared to be important for the immunoreactivity of TraM with polyclonal anti-TraM antiserum and could contain the principle antigenic determinants in this protein. 72 distinct missense mutations (including a 78% redundancy), covering 44% of TraM residues, clustered into three regions (I, II, and...
III; Fig. 1), which defined four different phenotypes for oligomerization, DNA binding, cooperativity of DNA binding, and transfer. Mutations in all three regions were defective in binding to DNA fragments containing \( \text{sbmA} \), \( \text{sbmB} \), and \( \text{sbmC} \), suggesting that binding to these sites is required for TraM to function in autoregulation and F conjugation. However, the three regions of TraM contributed to DNA binding via different mechanisms.

Mutations within region I did not affect TraM tetramerization but decreased the binding affinity of TraM for \( \text{sbmABC} \), suggesting that this region participates in direct contact with the DNA (Table IV). Asparagine (Asn) is the third most common residue to form hydrogen bonds with bases and DNA backbones in protein-DNA complexes (26). The Asn residue at position 5 might interact with the negatively charged DNA; thus, replacement of this residue with an aspartic acid (Asp) could abolish TraM binding to \( \text{sbmABC} \) (Fig. 5B). When Asn-10 of TraM was replaced by Asp, the amino acid at the equivalent position in R1 TraM (27), the resulting mutant protein bound to \( \text{sbmABC} \) less efficiently (Fig. 5C), suggesting that Asn-10 contributes to the allelic specificity of the F-like TraMs for their

| Protein | 60 | 200 | 600 | 2000 | 6000 |
|---------|----|-----|-----|------|------|
| TraM    |    |     |     |      |      |
| F120L   |    |     |     |      |      |

| Protein | 60 | 200 | 600 | 2000 | 6000 |
|---------|----|-----|-----|------|------|
| V4A     |    |     |     |      |      |
| N10D    |    |     |     |      |      |
| A37V    |    |     |     |      |      |
| R48C    |    |     |     |      |      |

| Protein | 60 | 200 | 600 | 2000 | 6000 |
|---------|----|-----|-----|------|------|
| I109T   |    |     |     |      |      |
| F120S   |    |     |     |      |      |
| F121V   |    |     |     |      |      |
| F121S   |    |     |     |      |      |

| Protein | 60 | 200 | 600 | 2000 | 6000 |
|---------|----|-----|-----|------|------|
| 1109T   |    |     |     |      |      |
| F120S   |    |     |     |      |      |
| F121V   |    |     |     |      |      |
| F121S   |    |     |     |      |      |

**Fig. 5.** DNA Binding of TraM and its mutants as determined using electrophoretic mobility shift assays. Increased concentrations of TraM or its mutants were indicated above each figure. Each reaction used 40 nM DNA fragments containing all three TraM binding sites (\( \text{sbmABC} \)) or a single site (\( \text{sbmA} \)). A, binding of TraM or F120L to \( \text{sbmABC} \); B, binding of S79* or N5D to \( \text{sbmABC} \); C, binding of V4A, N10D, A37V, or R48C to \( \text{sbmABC} \); D, binding of I109T, F120S, F121V, or F121S to \( \text{sbmABC} \); E, binding of TraM, I109T, or F121V to \( \text{sbmA} \).
cognate DNA (28). This agreed with the previous finding that the first 24 aa residues of R1 TraM define its DNA binding specificity (15).

Mutations F35S and R48C in region II did not affect TraM tetramerization but decreased the binding affinity of TraM for sbmAABC, suggesting that these residues also directly contribute to TraM binding to DNA (Table IV). Some other mutations within region II affected TraM tetramerization with A37V and Q53L forming small amounts of dimers as well as tetramers (Table IV). Thus region II appears to define a dimerization domain near the N terminus. Mutations in this region would give aberrant dimers resulting from interactions involving the second dimerization domain nearer the C terminus (Fig. 4C) (29). The presence of a dimerization domain near the N terminus is supported by the ability of a TraM fragment of aa 2–55 to form dimers (13). Thus the region II mutations probably affected protein-protein interactions during tetramerization. The decreased affinity of A37V or Q53L for sbmAABC suggests that tetramerization is also important for binding to DNA.

Missense mutations within region III appeared to dramatically affect the quaternary structure of TraM. S89F, S95P, F100S, and S114P did not bind efficiently to the Mono S column under the conditions used in this work, suggesting that major structural perturbations occurred in these mutant proteins. I109T, F120S, F121V, and F121S appeared to form conformationally altered tetramers as well as smaller amounts of dimers as determined by analytical SEC (Fig. 4B and Table IV). This is consistent with the finding that a C-terminal fragment of F TraM formed tetramers (13). Because tetramers require at least two distinct dimerization domains in each monomer, the loss of the ability to form tetramers suggests that these mutations affected one of these domains. Defects in tetramerization or conformational changes in the tetramers appear to affect DNA binding by TraM as well as transfer (Fig. 5D and Table IV).

It could be argued that the major peak for F121S (or similar mutants) during analytical SEC contained trimers or aberrantly large dimers; however, other evidence suggests that this peak contained conformationally altered tetramers. First, because the N-terminal region of TraM exists as dimers rather than monomers (13), oligomerization of the TraM mutant proteins containing an intact N-terminal region should result in dimers and tetramers not trimers. Second, because S79*, which lacks the C-terminal 49 residues of TraM, formed a minor peak corresponding to tetramers (Fig. 4D), F121S, with only one amino acid substitution, would be expected to also form tetramers. Third, I109T, F120S, F121V, and F121S were eluted from the cation-exchange column at slightly lower salt concentrations than TraM (see “Experimental Procedures”), suggesting these mutations resulted in conformational changes.

Blue native polyacrylamide gel electrophoresis suggested that I109T and F121S formed more dimers than tetramers (Fig. 3), which was opposite to the results obtained by analytical SEC (Fig. 4B). Native gel samples were prepared from cells expressing levels of wild type or mutant TraM comparable to the physiological levels of TraM expressed by the F plasmid (16). The concentration of TraM on the gel was comparatively low, and TraM could only be detected by immunoblot. Although analytical SEC is usually accurate in estimating sizes of native protein complexes, because the potential effects of differences in charge are negligible, it could be affected by the high concentrations of protein in the sample applied to the column. Because TraM has a tendency to aggregate, this might favor the formation of tetramers via mass action. Thus, I109T and F121S probably form more dimers than tetramers with the equilibrium shifted toward tetramers if aggregation is a factor.

Mutations that decreased TraM immunoreactivity appear to be located within the first 47 aa of TraM (Fig. 2). Because prolines disrupt higher order structure, mutations such as L42P and L47P could alter the structure of TraM during immunoblot analysis. Alternatively, mutations in the first 47 aa might alter the immunogenicity of TraM with a consequent reduction in immunoreactivity to specific preparations of antiserum. Certainly R100 TraM, which differs from F TraM within the first 40 aa, reacts poorly with anti-F TraM antiserum, which agrees with this observation (data not shown). The N-terminal region also defines the DNA binding domain of the different alleles of TraM expressed by the various F-like plasmids (F, R1, and P307 (14, 15, 30)), suggesting that these residues are exposed on the surface of the protein and form contacts with the DNA.

Nearly normal repression for P_traM was observed when I109T was overexpressed from P_traM, indicating that the increased dosage of I109T compensated for its inability to autoregulate the traM promoter. This agreed with previous observations that expressing mutants of an autoregulatory gene product from its native promoter compromises its characterization because of the compensatory effect of protein overexpression (16). This result also suggested that a mass action effect of I109T at P_traM resulted in repression even though I109T was defective for tetramerization. However, overexpression of I109T from P_traM could not complement pOX38-MK3 for conjugative DNA transfer, indicating that F conjugation requires the quaternary structural integrity of TraM.

This work has defined three domains of TraM involved in tetramerization or DNA binding, two properties important for TraM function in autoregulation and F conjugation. The N-terminal region of TraM (region I) is required for DNA binding and defines its allelic specificity, agreeing with previous results showing that the N terminus of TraM from F-like R1 is responsible for specific DNA binding (15, 30). Tetramerization of F plasmid TraM increases the binding affinity of TraM for its cognate sites, consistent with the finding that R1 TraM binds to DNA as a tetramer (14). Involvement of extensive regions (II and III) in oligomerization presumably ensures efficient tetramerization of TraM, which could explain why monomers and tetramers but not dimers were detectable in the equilibrium studies of Miller and Schildbach (13). The C-terminal domain (region III) of TraM is not only important for tetramerization but also for the cooperativity of binding to DNA, because it appears to have a role in defining the order that TraM binds to the three different sites, sbmA, -B, and -C (10).

We propose that TraM forms a DNA binding domain at the N terminus (region I) through tetramerization at regions II and III. Through cooperative binding, TraM occupies all three sites at oriT during relaxosome formation. The F relaxosome normally resides at the cell center or quarter position during exponential growth (31) when there is no occasion for conjugal DNA synthesis (7). After donor and recipient cells contact each other, TraM might connect the relaxosome to the trans-ferosone through interactions with the coupling protein, TraD (32). TraM-TraD interactions might further change the binding properties of TraM at oriT, causing localized denaturation between sbmAABC and the nic site. This localized melting at oriT could provide a region of single-stranded DNA that is required for inducing DNA helicase activity of TraI to further unwind the DNA for conjugal transfer (33). In this manner, TraM might indeed act as a “signaling factor” to trigger conjugal DNA transfer as proposed by Willetts and Wilkins (1).

Acknowledgments—We thank Dr. Frank Nargang and Suzanne Hoppins for assistance in blue native gel electrophoresis. We also thank Jan Manchak and Troy Locke for general technical assistance.
REFERENCES

1. Willetts, N. S., and Wilkins, B. (1984) Microbiol. Rev. 48, 24–41
2. Lanka, E., and Wilkins, B. M. (1995) Annu. Rev. Biochem. 64, 141–169
3. Sherman, J. A., and Matson, S. W. (1994) J. Biol. Chem. 269, 26220–26226
4. Ippen-Ihler, K. A., and Skurray, R. A. (1993) in Bacterial Conjugation (Clewell, D. B., ed) pp. 23–52, Plenum Publishing, New York
5. Frost, L. S., Ippen-Ihler, K., and Skurray, R. A. (1994) Microbiol. Rev. 58, 162–210
6. Achman, M., Willetts, N., and Clark, A. J. (1972) J. Bacteriol. 110, 831–842
7. Sherman, J. A., and Matson, S. W. (1994) J. Biol. Chem. 269, 26220–26226
8. Everett, R., and Willetts, N. (1980) J. Mol. Biol. 136, 129–150
9. Di Laurenzio, L., Frost, L. S., and Paranchych, W. (1992) Mol. Microbiol. 6, 2951–2959
10. Fekete, R. A., and Frost, L. S. (2002) J. Biol. Chem. 277, 16705–16711
11. Penfold, S. S., Simon, J., and Frost, L. S. (1996) Mol. Microbiol. 20, 549–558
12. Fu, Y. H., Tsai, M. M., Luo, Y. N., and Deonier, R. C. (1991) J. Bacteriol. 110, 831–842
13. Kingsman, A., and Willetts, N. (1978) J. Mol. Biol. 122, 287–300
14. Miller, D. L., and Schildbach, J. F. (2003) J. Biol. Chem. 278, 10400–10407
15. Miller, D. L., and Schildbach, J. F. (2000) J. Bacteriol. 182, 4022–4027
16. Lu, J., Fekete, R. A., and Frost, L. S. (2003) Mol. Genet. Genomics 269, 227–233
17. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, 2nd Ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
18. Moore, D., Wu, J. H., Kathir, P., Hamilton, C. M., and Ippen-Ihler, K. (1987) J. Bacteriol. 169, 3994–4002
19. Willetts, N. S., and Finnegan, D. J. (1970) Genet. Res. 16, 113–122
20. Hanahan, D. (1983) J. Mol. Biol. 166, 557–580
21. Miller, J. H. (1972) Experiments in Molecular Genetics, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
22. Lu, J., Manchak, J., Klimke, W., Davidson, C., Firth, N., Skurray, R. A., and Frost, L. S. (2002) Plasmid 48, 24–37
23. Schagger, H., and von Jagow, G. (1991) Anal. Biochem. 199, 223–231
24. Schagger, H., Cramer, W. A., and von Jagow, G. (1994) Anal. Biochem. 217, 220–230
25. Lane, D., Prenkti, P., and Chandler, M. (1992) Microbiol. Rev. 56, 509–528
26. Luscombe, N. M., Laskowski, R. A., and Thornton, J. M. (2001) Nucleic Acids Res. 29, 2860–2874
27. Finlay, B. B., Frost, L. S., and Paranchych, W. (1986) J. Bacteriol. 166, 368–374
28. Fekete, R. A., and Frost, L. S. (2000) J. Bacteriol. 182, 4022–4027
29. Hagel, L. (1998) in Current Protocols in Protein Science (Coligan, J. E., Dunn, B. M., Ploegh, H. L., Speicher, D. W., and Wingfield, P. T., eds) pp 8.3.1–8.3.30, John Wiley & Sons, New York
30. Kulpmier, G., Schwab, M., Hogenauer, G., Koraimann, G., and Zechar, E. L. (1998) J. Mol. Biol. 275, 81–94
31. Niki, H., and Hirose, S. (1997) Cell 90, 951–957
32. Schagger, H., Cramer, W. A., and von Jagow, G. (1994) Anal. Biochem. 217, 220–230
33. Cstkovits, V. C., and Zechar, E. L. (2003) J. Biol. Chem. 278, 48696–48703
34. Short, J. M., Fernandez, J. M., Surge, J. A., and Huse, W. D. (1988) Nucleic Acids Res. 16, 7583–7600
35. Tabor, S., and Richardson, C. C. (1985) Proc. Natl. Acad. Sci. U.S.A. 82, 1074–1078