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Construction and Analysis of F Plasmid *traR*, *trbJ*, and *trbH* Mutants

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F plasmid derivatives carrying *kan* insertion mutations in the transfer region genes *traR*, *trbJ*, and *trbH* were constructed. Standard tests indicated that these loci are not essential for F pilus production or F transfer among *Escherichia coli* K-12 hosts. Among the *traR* and *trbH* mutants tested, the orientation of the *kan* cassette had no effect on the mutant phenotype. In each case, there was no significant effect on the appearance of F pili, the transfer frequency, or the plating efficiency of F-pilus-specific phages. The *trbJ* insertion carrying a *kan* gene oriented in the direction opposite to *tra* transcription had very little effect on phage sensitivity but markedly reduced the plasmid transfer efficiency. However, the *kan* insertion mutation at the same site, in the *tra* orientation, did not seem to affect either property. Analysis of clones carrying *trbJ* sequences regulated by a phage T7 promoter showed that *trbJ* expresses an approximately 11-kDa protein product. The TrbJ protein was not expressed from clones carrying a *kan* insertion or stop codon linker insertion in the *trbJ* sequence. However, it was expressed from clones that did not include sequences at the beginning of the 113-codon open reading frame in this region. Our data indicated that translation of *trbJ* must be initiated at the more distal GUG codon in this frame. This would result in expression of a 93-amino-acid polypeptide.

Genetic analysis of the F conjugation system originally depended on the isolation of F transfer-deficient mutants. Early studies of this type led to the identification of 17 F *tra* genes and characterized their functions in conjugation (6, 18). More recently, the existence of a number of additional F *tra* region genes has been reported as a result of DNA sequence and product analysis. To test the contribution of these and other loci to F transfer functions, our laboratory has been constructing and analyzing F plasmid derivatives carrying site-specific insertion mutations in *tra* region sequences (7, 9–12, 14). In the approach used, a selectable marker gene is first inserted into a restriction site within the cloned *tra* sequence on a multicopy plasmid. Donors carrying the transfer-proficient F derivative, pOX38 (4), are then mated with cells carrying the mutated clone. After in vivo homologous recombination between the plasmids, mutant pOX38 derivatives can be transferred to another recipient since coresident wild-type plasmids will complement their defects. Transconjugants carrying these mutant plasmids can be identified after selection for transfer of the marker gene used to make the insertion mutation.

We have used this method to construct and characterize pOX38 mutants that carry insertion mutations in *traR*, *trbJ*, and *trbH*. Bacterial strains and methods employed have been described in detail previously (9–12). Plasmids constructed for this study are described in Table 1. Vectors included pBR322 (16), pBS/KS(+) (Stratagene, La Jolla, Calif.), pKI497 (11), and pUC8 (17). Kanamycin resistance cassettes were restriction fragments from pUC4K and pUC4KISS (Molecular Biology Division, Pharmacia Inc., Piscataway, N.J.). The stop codon linker (CTAGTCTA GACTAG), which contains an *Xba*I site and three nonsense codons, was purchased from New England Biolabs, Inc., Beverly, Mass. To obtain pOX38 derivatives, triparental matings were performed by mixing the pOX38 donor strain, RD17, with a derivative of strain XK5456 (F^- *rpsE*) carrying the *kan* insertion mutation on a multicopy plasmid and the final recipient strain, VL584 [F^- Δ(*uxu-fimD*) *rpsL*] (10, 11). Kanamycin-resistant, streptomycin-resistant transconjugants were selected and screened to identify those lacking an antibiotic resistance associated with the multicopy vector sequence. The structure of all pOX38 derivatives was subsequently confirmed by restriction fragment analysis.

***traR*.** The *traR* gene was originally identified through detection of its 9-kDa product and mapped within a *Sma*I fragment spanning the region between *traV* and *traC* (15). As indicated in Fig. 1, pKI353 is a clone carrying this *traR-Sma*I fragment. Insertions of a kanamycin resistance gene cassette (*kan*) were made at the unique *Sph*I site, known to be in *traR* (15). Two plasmids, carrying *kan* inserts oriented in the same direction as *traR* (pKI354) or opposite direction to *traR* (pKI355), were obtained. After in vivo recombination, the corresponding mutant derivatives, pOX38-*traR354* and pOX38-*traR355*, were identified. Both of these plasmids were able to transfer at a wild-type mating frequency, and cells carrying either of the pOX38-*traR* mutants were fully sensitive to F-pilus-specific phages (Table 2). Electron microscopy also showed that the F pili on these cells were normal in appearance.

***trbJ*.** DNA sequence analysis had revealed the existence of a 113-codon open reading frame (ORF) (*orf113*) in the *trbB-trbF* region (20). Although the methionine codon (AUG) at the beginning of this ORF was preceded by a possible ribosome binding sequence (AGGGGG), its location within the upstream *trbB* gene suggested that translation might actually be initiated at the 21st codon (GUG) in the ORF (Fig. 2). This valine codon was located only 8 nucleotides before the *trbB* stop codon and was also preceded by a reasonable ribosome binding sequence (GGAGG). In this case, a 93-amino-acid product (of *orf93*) would be expressed. However, analysis of proteins labeled in maxicells had not

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TABLE 1. Plasmids

Plasmid	Description
pKI288	3.0-kb *trbH EcoRV-HpaI* fragment cloned in pBR322 *EcoRV* site; *trbH* is in the *tcy* promoter orientation
pKI290	1.2-kb *kan-BamHI* fragment from pUC4K inserted in the pKI288 *BclI* site; *kan* is in the same orientation as *trbH*
pKI291	1.2-kb *kan-BamHI* fragment from pUC4K inserted in the pKI288 *BclI* site; *kan* is in the opposite orientation to *trbH*
pKI353	1.1-kb *traR-SmaI* fragment cloned in pUC8 *SmaI* site; *traR* is in the *lac* promoter orientation
pKI354	1.2-kb *kan-SphI* fragment from pUC4KISS inserted in the pKI353 *SphI* site; *kan* is in the same orientation as *traR*
pKI355	1.2-kb *kan-SphI* fragment from pUC4KISS inserted in the pKI353 *SphI* site; *kan* is in the opposite orientation to *traR*
pKI466	0.6-kb *trbJ HincII-AccI* fragment cloned in pKI497 *HincII* site; *trbJ* is in the opposite orientation to the *tcy* promoter
pKI478	DNA sequence of pKI466 altered by site-directed mutagenesis to create a *PstI* site 370 bp distal to the *HincII* site
pKI479	1.2-kb *kan-PstI* fragment from pUC4K inserted in the pKI478 *PstI* site; *kan* is in the same orientation as *trbJ*
pKI480	1.2-kb *kan-PstI* fragment from pUC4K inserted in the pKI478 *PstI* site; *kan* is in the opposite orientation to *trbJ*
pKM50	0.3-kb *trbJ-RsaI* fragment cloned in pBS/KS(+) *SmaI* site; *trbJ* is in the *kan* promoter orientation
pKM54	0.6-kb *trbJ HincII-AccI* fragment cloned in pBS/KS(+) *SmaI* site; *trbJ* is in the *lac* promoter orientation
pKM55	0.6-kb *trbJ-PstI HincII-AccI* fragment from pKI478 cloned in pBS/KS(+) *SmaI* site; *trbJ* is in the *lac* promoter orientation
pKM56	1.2-kb *kan-PstI* fragment from pUC4K inserted in the pKM55 *PstI* site; *kan* is in the same orientation as *trbJ*
pKM57	1.2-kb *kan-PstI* fragment from pUC4K inserted in the pKM55 *PstI* site; *kan* is in the opposite orientation to *trbJ*
pKM63	*XbaI* stop linker inserted in the pKM50 *BspMI* site

detected either of the predicted products (20). To further examine the products and function of this region, we constructed the clones and mutations depicted in Fig. 2.

To test whether *orf113* and/or *orf93* expressed protein products, we cloned two different DNA segments under the control of the T7 promoter on vector pBS/KS(+). T7 RNA polymerase expression was induced in the host XK100, and plasmid products were labeled with [^{35}S]methionine in the presence of rifampin and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and autoradiography as described previously (9). As shown in Fig. 3, pKM54, which contained a *HincII-AccI* fragment including both of the putative translational start sites, expressed a polypeptide with an apparent molecular mass of 11 kDa (lane 3). This product was not expressed by pKM56 (Fig. 3, lane 1) or pKM57 (lane 2), which are analogous plasmids carrying *kan* inserts that interrupt the ORF. However, the 11-kDa protein was also expressed by pKM50 (Fig. 3, lane 4), which includes all of *orf93* but not the upstream AUG codon. Plasmid pKM63, in which we had inserted a stop codon linker at the *BspMI* site in the ORF, did not express the protein (Fig. 3, lane 5). Since pKM50 did express the 11-kDa protein, this product must be encoded by *orf93* and stem from translational initiation at the valine codon. We have named the *orf93* locus *trbJ*. The *trbJ* DNA sequence (20) indicates that TrbJ protein amino acid residues 6 to 29 and 42 to 67 may be membrane spanning and that TrbJ may localize in the inner membrane of the cell.

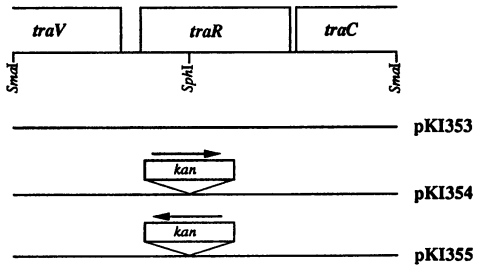

FIG. 1. Construction of *traR* mutations. A map of the 1.1-kb *SmaI* fragment carried by pKI353. Boxes show the positions of the gene sequences indicated; open-ended boxes indicate that only a portion of the sequence is present. Lines below the map show the *tra* DNA carried by various plasmid clones. On these, boxes (not to scale) and arrows mark the position and direction of transcription of *kan* inserts. As drawn, *tra* and *trb* gene transcription is from left to right.

F derivatives carrying *kan* insertion mutations in the *trbJ* sequence were also constructed and characterized. In this case, we began by constructing pKI466, which also contains the *HincII-AccI* fragment from this *tra* region (Fig. 2). At the time, no unique restriction enzyme sites had been identified within *trbJ*, so we used site-directed mutagenesis (13) to change the DNA sequence of the 29th and 33rd nucleotides downstream from the *trbJ* translational start site and create a *PstI* site (pKI478). The *kan* insertions in this site on pKI479 and pKI480 are oriented in the same direction as and opposite direction to *trbJ*, respectively (Fig. 2). Both were crossed with pOX38, and corresponding pOX38 *trbJ* mutant plasmids were isolated. pOX38-*trbJ479* showed the same transfer efficiency and F-specific-phage sensitivity as the wild-type control (Table 2). The pOX38-*trbJ480* strain could transfer DNA only at about 0.02% of the wild-type transfer frequency. Interestingly, R17 and M13 still plated very

TABLE 2. Phenotypes of pOX38 derivatives carrying mutations in *traR*, *trbJ*, and *trbH*

Plasmid[a]	Transfer frequency[b]	Sensitivity[c] to phages:	
		M13, f1, and fd	f2, Qβ, and R17
pOX38-Km[d]	82.0	S	S
pOX38-*traR354*	96.0	S	S
pOX38-*traR355*	87.0	S	S
pOX38-*trbJ479*	75.0	S	S
pOX38-*trbJ480*	0.015	S[e]	S[e]
pOX38-*trbH290*	88.0	S	S[f]
pOX38-*trbH291*	90.0	S	S[f]

[a] In the host VL584.
[b] Number of Km^r transconjugants per 100 donor cells in the mating mixture. The recipient, XK1200, and mating conditions have been described elsewhere (11).
[c] S, sensitive to all phages on spot tests, with (except as noted) R17 and M13 plaque morphology and plating efficiency which did not significantly differ from those of pOX38-Km controls.
[d] Wild-type control; *kan* is in the pOX38 *HindIII* site (3).
[e] R17 plaques were turbid; M13 plated at one-third wild-type efficiency.
[f] R17 plaques were somewhat smaller than usual.

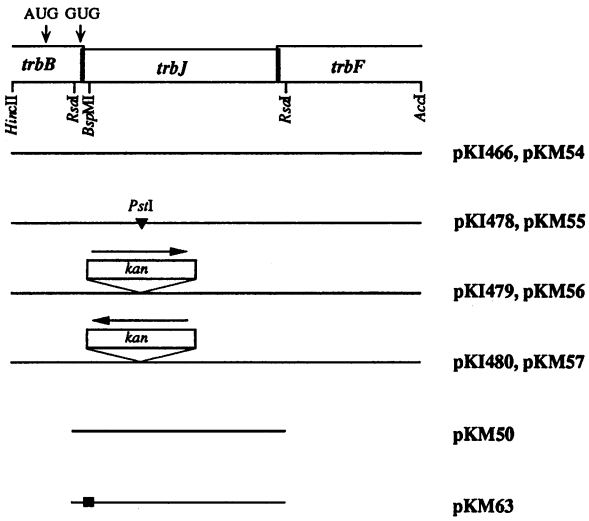

FIG. 2. Construction of *trbJ* clones and mutations. The region contained in the 0.6-kb *Hin*cII-*Acc*I fragment carried by pKI466 and pKM54 is depicted. Vertical arrows indicate the positions of the two in-frame start codons with the potential to initiate translation of *orf113* or *orf93* (*trbJ*). The black triangle shows the position of a *Pst*I site introduced by site-directed mutagenesis, and the black rectangle shows the position of a stop codon linker insertion. Pairs of pKI and pKM plasmids carry the same fragment in different vectors. Other features are as described for Fig. 1.

efficiently on this strain, although the R17 plaques had an unusually turbid appearance. Since the only difference between the two *trbJ* mutations is the orientation of the *kan* insert, the pOX38-*trbJ480* phenotype cannot result from loss of TrbJ. Instead, these properties may stem from a polarity effect or reflect interference from an anti-*tra* RNA transcript originating from the upstream-oriented *kan* promoter in

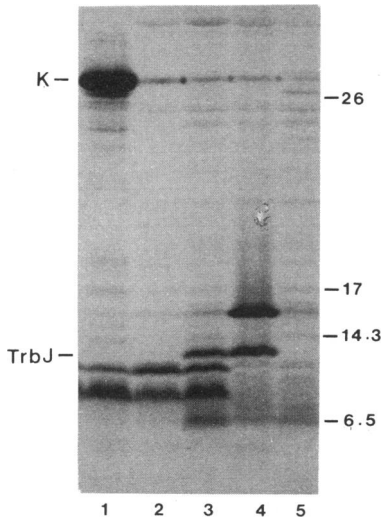

FIG. 3. Identification of the *trbJ* product. An autoradiograph of samples fractionated by SDS-PAGE is shown. Samples were of XK100 strains carrying pKM56 (lane 1), pKM57 (lane 2), pKM54 (lane 3), pKM50 (lane 4), and pKM63 (lane 5). These were labeled after induction of T7 RNA polymerase and rifampin treatment. The positions of the TrbJ protein, the *kan* product (K), and molecular size markers (in kilodaltons) are marked.

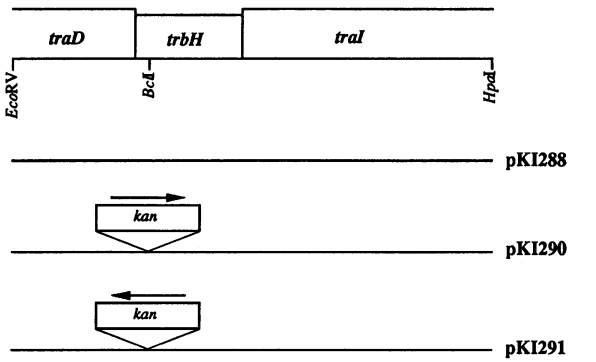

FIG. 4. Construction of *trbH* mutations. The map shows the 3.0-kb *Eco*RV-*Hpa*I fragment carried by pKI288. Lines below the map show the position and orientation of the *kan* inserts in pKI290 and pKI291. Other features are as described for Fig. 1.

trbJ480. Although *tra* region *kan* inserts in either orientation do not usually exhibit any obvious polarity (7, 9–12, 14), a similar phenotypic difference related to the direction of a *kan* insert has been observed in two other cases (pOX38-*artA249* and pOX38-*traP470*) (7, 8).

trbH. The DNA sequence reported by Bradshaw et al. (2) included another previously uncharacterized ORF, *trbH*, located in the interval between *traD* and *traI*. Its product has been predicted to be a 26.2-kDa polypeptide. The sequences of the *trbH* clones we constructed and the *kan* insertions we made are diagramed in Fig. 4. The *kan* cassette was inserted at the unique *Bcl*I site in pKI288. It is oriented in the *trbH* direction in pKI290 and in the anti-*trbH* direction in pKI291. After crossing these *trbH* mutations onto pOX38, we found that both derivatives (pOX38-*trbH290* and pOX38-*trbH291*) transferred very efficiently and conferred sensitivity to F-pilus-specific phages (Table 2). Previous work has shown that *traD* amber mutations and Hfr deletions that extend through *trbH* into *traD* affect sensitivity to RNA phages in the f2, R17 family (1, 5). Cells carrying the plasmid R100, which does not contain a *trbH* gene (21), are also more resistant to these phages (17). Therefore, it seemed possible that *trbH* mutations would affect RNA phage sensitivity. We did observe that f2 and R17 plaques were somewhat smaller on pOX38-*trbH* mutant strains. Nevertheless, no substantial deviation in plaque count was observed in comparison with the pOX38-Km control, whereas the plating efficiency on an R100-1 host was reduced by a factor of 20. When the pOX38-*trbH* strains were viewed by electron microscopy, both pilus-specific RNA phage adsorption and F piliation appeared to be normal.

We also cloned *trbH* in vector pBS/KS(+) and attempted to detect *trbH* expression from the T7 promoter on this plasmid. The experiment was performed as described for *trbJ*. However, no product in the vicinity of 26 kDa was obvious on the autoradiogram obtained (data not shown).

The phenotypes of the pOX38-*traR*, pOX38-*trbJ*, and pOX38-*trbH* mutants we constructed indicate that, under standard conditions, F does not require these three genes for conjugation or F pilus production. Previous studies have shown that there are also other F *tra* region genes that are dispensable for F piliation and transfer in *Escherichia coli* K-12 hosts. Insertion mutations in *trbA*, *trbB*, *trbE*, and *artA*, similarly, do not appear to alter the efficiency of transfer or F-pilus-specific phage infection (7, 10). Nevertheless, several features suggest that such loci may be involved

in transfer processes. With the exception of *artA*, all seem likely to require induction of the *tra* operon for maximal expression, and in most cases, sequence overlaps suggest translational coupling with neighboring genes (2, 10, 20). The products of several are known to locate in the inner membrane (TrbA and TrbE) or periplasm (TrbB), and the predicted locations of TrbJ and TrbH suggest that they too could be part of a complex of transfer proteins in the cellular envelope. If so, they may affect more subtle features of F piliation or mating kinetics, or they may play a more critical role in a different host or under more natural physiological conditions. The plasmids we have constructed should be useful for assessing such possibilities.

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