The Mating Pair Formation System of Plasmid RP4 Defined by RSF1010 Mobilization and Donor-Specific Phage Propagation

MONIKA LESSL, DIETMAR BALZER, KERSTIN WEYRAUCH, AND ERICH LANKA*

Max-Planck-Institut für Molekulare Genetik, Abteilung Schuster, Ihnestr. 73, D-14195 Berlin, Germany

Received 9 February 1993/Accepted 9 August 1993

Transfer functions of the conjugative plasmid RP4 (IncP α) are distributed among distinct regions of the genome, designated Tra1 and Tra2. By deletion analyses, we determined the limits of the Tra1 region, essential for intraspecific *Escherichia coli* matings. The Tra1 core region encompasses approximately 5.8 kb, including the genes *traF*, *-G*, *-H*, *-I*, *-J*, and *-K* as well as the origin of transfer. The *traM* gene product, however, is not absolutely required for conjugation but significantly increases transfer efficiency. To determine the transfer phenotype of genes encoded by the Tra2 core region, we generated a series of defined Tra2 mutants. This revealed that at least *trbB*, *-C*, *-E*, *-G*, and *-L* are essential for RP4 conjugation. To classify these transfer functions as components of the DNA transfer and replication (Dtr) or of the mating pair formation (Mpf) system, we analyzed the corresponding derivatives with respect to mobilization of IncQ plasmids and donor-specific phage propagation. We found that all of the Tra2 genes listed above and the *traG* and *traF* genes of Tra1 are required for RSF1010 mobilization. Expression of *traF* from Tra1 in conjunction with the Tra2 core was sufficient for phage propagation. This implies that the TraG protein is not directly involved in pilus formation and potentially connects the relaxosome with proteins enabling the membrane passage of the DNA. The proposed roles of the RP4 transfer gene products are discussed in the context of virulence functions encoded by the evolutionarily related Ti T-DNA transfer system of agrobacteria.

The study of conjugative DNA transfer of IncP plasmids gains increasing interest, since transfer of these plasmids to a wide range of remotely related microorganisms (for review, see references 21, 23, 65, and 76), even including yeasts (25), was observed. In addition, several findings provide evidence for a close relationship between RP4-mediated bacterial conjugation and T-DNA transfer from agrobacteria to plant cells (37, 39, 49, 72, 81). These similarities are underscored by recent data demonstrating that the *Agrobacterium tumefaciens* pTi Vir region can mobilize the small non-self-transmissible IncQ plasmid RSF1010 among agrobacteria (7) in a manner resembling the mobilization of these plasmids by IncP plasmids (16, 78). However, it is noteworthy that mobilization frequencies obtained by the Ti system are extremely low.

Functions responsible for RP4 transfer are encoded by two distinct regions of the genome, designated Tra1 and Tra2. The Tra1 region encompasses the origin of transfer (*oriT*) as well as functions responsible for the cleaving-joining process at the nick site (*traI*, *-J*, and *-K*; for review, see references 23 and 76). The *traI* and *traJ* gene products are required for relaxosome formation as well as for site- and strand-specific nicking at *oriT*, by which TraI becomes covalently associated with the 5' end of the DNA strand destined for transfer to the recipient cell (51). *traK* and *traI* are specificity determinants on the basis of the observation that their gene products are not interchangeable between plasmids of different IncP subgroups (i.e., IncP α and IncP β) (18). Both genes encode specific DNA-binding proteins forming highly ordered nucleoprotein structures within the transfer origin (79, 80). Interestingly, Rohrer and Rawlings (54) recently identified a series of Mob proteins of plasmid pTF-FC2, exhibiting extensive sequence similarities to the RP4 Tra1 proteins TraI, *-J*, *-K*, and *-L*. Plasmid pTF-FC2 is a nonconjugative, broad-host-range plasmid of *Thiobacillus ferrooxidans*, which can be mobilized efficiently by IncP plasmids.

The arrangement of these *mob* genes resembles the gene organization found in RP4 (81); in both plasmids, these genes flank the origin of transfer. This suggests that both transfer systems may have evolved from a common ancestor.

However, Tra1 genes are not only involved in DNA processing, but are also involved in the formation of mating pairs between conjugating cells (3, 4). This was shown by the use of a binary plasmid system, in which Tra1 and Tra2 were separated by subcloning and expressed from compatible replicons to reconstitute a transfer system functional in *Escherichia coli* (36). Both Tra1 and Tra2 were essential to mobilize the IncQ plasmid RSF1010 and for propagation of donor-specific bacteriophages. Recently, Waters et al. (73) identified the *traF* gene product as one Tra1 component essential for phage propagation. However, it remained unclear whether additional Tra1 functions are required for this ability.

The second section of RP4 required for conjugative DNA transfer is the Tra2 region, which probably encodes most of the functions involved in formation of mating pairs. Delimitation of Tra2 by deletion studies identified an 11.23-kb Tra2 core region essential for matings between *E. coli* cells. Subsequent nucleotide sequence analysis revealed 12 open reading frames (ORFs) within this region, designated *trbA* to *-L* (29, 37, 38, 41, 68). Ten of these ORFs were tentatively verified by T7 Φ 10-controlled expression in *E. coli* (*trbA*, *-C*, *-E*, *-F*, *-G*, *-H*, *-I*, *-J*, *-K*, *-L* [37]), *trbA* and *trbB* being verified by specific expression in a *tac* promoter system (29, 41). The predicted amino acid sequences of most of the Tra2 proteins disclosed features characteristic of membrane proteins, such as potential signal sequences and membrane-spanning regions (37). Thus, a membrane location for these proteins was suggested. Similarities of Tra2 gene products to VirB proteins of the *A. tumefaciens* Ti plasmid strengthened this suggestion, since a proposed role for VirB proteins is to form a membrane-spanning pore or channel for the exit of the T-DNA from the bacterial cell to the plant cell (12).

Transcription of Tra2 seems to be initiated at two promoter

* Corresponding author.

sites located at the *trfA* proximal region of Tra2 (36). Both were verified by RNA-polymerase binding; the promoter upstream of *trbB* was also confirmed by primer extension analysis (68). Initial studies identified the key repressor proteins encoded by the *korA-korB* control region (for review, see references 6, 14, 65, and 66) as well as TrbA, located at the *trfA* proximal end of Tra2, as transcriptional regulators of the Tra2 region (29).

Since the extent of the Tra2 core region required for *E. coli* matings was determined recently (36), we now present the limits of Tra1. Using RSF1010 mobilization and donor-specific phage propagation, we identified Tra1 functions responsible for the contact between donor and recipient cells. To continue with the analysis of the Tra2 region, we generated six defined Tra2 mutants by insertion of a so-called multiple reading frame insertion (MURFI) linker (52) in respective ORFs. This method was chosen to minimize transcriptional polar effects (73) on downstream genes. Accordingly, we found *trbB*, *-C*, *-E*, *-G*, and *-L* to be essential for RP4 transfer, IncQ mobilization, and phage propagation. To evaluate the role of *trbA* in conjugative transfer, a Tra2 derivative was constructed by replacing *trbA* and the entire Tra2 regulatory region by a LacI-regulated *tac* promoter. The resulting derivative exhibited a transfer-proficient phenotype, indicating that *trbA* is not an essential transfer gene. Induction of the LacI-controlled transcription resulted in strong overexpression of Tra2 proteins, which were even visible on Coomassie blue-stained sodium dodecyl sulfate (SDS)-polyacrylamide gels. This underscores the hitherto-gained impression that the Tra2 region is organized as a single transcriptional unit (36–38, 68).

MATERIALS AND METHODS

Nomenclature. RP4 Tra1 ORFs were designated *tra* (81), and Tra2 ORFs were designated *trb* (37).

Strains, phages, and plasmids. *E. coli* strains used in this study were HB101 (10), a spontaneous nalidixic acid-resistant derivative of HB101 (HB101 Nx^r); SCS1, a DH1 derivative (24); a spontaneous rifampin-resistant derivative of SCS1 (SCS1 Rif^r); and S17-1, a strain which carries RP4 integrated into the chromosome (58). Cells were grown in yeast-tryptone (YT) medium buffered with 25 mM 3-(*N*-morpholino)propanesulfonic acid (sodium salt; pH 8.0) and supplemented with 0.1% glucose and 25 µg of thiamine-hydrochloride per ml. Antibiotics were used at the following concentrations: ampicillin (sodium salt), 100 µg/ml; chloramphenicol, 10 µg/ml; kanamycin sulfate, 30 µg/ml; nalidixic acid (sodium salt), 30 µg/ml; rifampin, 50 µg/ml. To select ampicillin-resistant (Ap^r) transconjugants, methicillin (2 mg/ml) was added to the medium to inhibit the background growth of sensitive cells. Phages Pf3 (61), PRR1 (45, 46), and PRD1 (44) were propagated as described previously (61). The plasmids used are listed in Table 1.

Test for phage susceptibility. Bacteria were seeded onto the surface of selective medium with YT extract soft agar (0.7%). Dilutions of the phage stocks were spotted on these plates and incubated overnight at 37°C. Efficiency of plating (EOP) was determined by comparing PFUs of various strains with those of an RP4-containing strain. No differences were detected, when the EOPs obtained by either SCS1 or HB101 as a host strain for plasmid RP4 were compared.

DNA techniques. Standard molecular cloning techniques were performed as described previously (55). For plasmid DNA sequencing, templates were purified by ethidium bromide-CsCl gradients and denatured by alkali (0.2 M NaOH) (55). DNA sequencing was carried out with T7 Sequenase

according to the sequencing protocol (Pharmacia). Synthetic oligonucleotides 18 to 25 nucleotides in length were applied as specific primers to examine correct insertion of the MURFI linker.

Generation of Tra2 mutants. For the construction of RP4 Tra2 mutants, a MURFI linker (52) was used (CTAGTCTA GACTAG). This linker was inserted into appropriate restriction sites located in Tra2 ORFs. Before linker insertion, the cleaved DNA was treated with T4 DNA polymerase to obtain blunt ends. The template DNA for insertion of the MURFI linker was pDB1756Δ1.1.3, except for the linker insertion in *trbC*, in which pDB1756Δ1.1 was used (Table 1).

Conjugations. Qualitative spot matings were carried out by inoculating 10-µl samples of serial dilutions of the donor cultures onto the surface of selective medium previously seeded with 100 µl of the recipient culture (HB101 Nx^r or SCS1 Rif^r). Quantitative filter matings were performed as described previously (36).

Expression of Tra2 proteins. Cells were grown at 37°C in YT medium to an *A*₆₀₀ of 0.5; IPTG (isopropyl-β-D-thiogalactopyranoside [1 mM]) was added, and incubation continued for 4 h. The cells were then harvested by centrifugation and lysed (1 *A*₆₀₀ unit) in 30 µl of cracking buffer (15% [wt/vol] glycerol, 100 mM Tris-HCl [pH 6.9], 5% [wt/vol] SDS, 1 M 2-mercaptoethanol). The suspension was incubated for 5 min at 60°C, followed by 2 min at 100°C, and then was centrifuged (80,000 × *g*, 30 min) at 25°C to clarify the extract. Appropriate aliquots (1 to 10 µl) of the cell extracts were electrophoresed on denaturing polyacrylamide gels containing 0.1% SDS. After gel electrophoresis, proteins were stained in 0.25% Coomassie brilliant blue R-250 (Serva)–50% (vol/vol) methanol–9% (vol/vol) acetic acid.

Solid-phase immunoassay. Proteins were visualized by immunoblotting as described previously (36, 69).

RESULTS

Definition of the essential Tra1 section for intraspecific *E. coli* matings. In order to determine the section of the Tra1 region essential for intraspecific *E. coli* matings, we performed a deletion analysis of the RP4 Tra1 region with the kanamycin-resistant (Km^r) Tra1 plasmid pVWDG23110 (Table 1 and Fig. 1). This plasmid, which has a pMB1 type replicon, carries the entire Tra1 region and the *korA-korB* operon. pVWDG23110 deletion derivatives used to delimit the Tra1 core region are shown in Fig. 1. Transfer of these derivatives was tested in the binary plasmid system (36), in which Tra2 functions are provided by pDB1756Δ1.1 in *trans*. Since stable maintenance of pDB1756Δ1.1 requires functions of the *korA-korB* operon (1, 14, 36, 65), this region was kept intact in each of the Tra1 derivatives. For the mating experiments, an Nx^r strain of HB101 was used as a recipient. Since only the *oriT*-carrying Tra1 plasmid can be transferred, transconjugants were selected on medium containing nalidixic acid and kanamycin. Transfer frequencies of the Tra1 deletion derivatives are listed in Table 2.

Plasmid pVWDG23110Δ10, carrying Tra1 genes *traF* to *traO*, was still transfer proficient, whereas the derivative pVWDG23110Δ11 was transfer deficient. The derivative pVWDG23110Δ11 lacks *traF*, indicating that *traF* encodes an essential RP4 transfer function. This is consistent with data obtained by Waters et al. (73), who found that insertion of a MURFI linker in *traF* resulted in a complete loss of transfer ability under the conditions used here. Most of the genes of the primase operon (40), namely *traA* to *-E*, are apparently unnecessary for *E. coli* × *E. coli* matings, since transfer frequencies

TABLE 1. Plasmids used in this study

Plasmid	Description	Reference or source
pVWDG23110	pBR329Ω(RP4 <i>TraI</i>)	36
pDB1756Δ1.1	pGZ119EHΩ(RP4 <i>EarI-PstI</i> <i>Tra2</i> -containing fragment, 12.9 kb), <i>Cm</i> ^r	36
pDB1756Δ1.1.3	pGZ119EHΩ(RP4 <i>EarI-NotI</i> 11.2-kb fragment), <i>Cm</i> ^r	36
pDB1756Δ1.1.2	pGZ119EHΩ(RP4 <i>EarI-NotI</i> [12.1-kb fragment]), <i>Cm</i> ^r	This work
pML123	pDB1756Δ1.1.2 Δ(RP4 <i>EarI-XmnI</i> , 0.9-kb fragment)Ω(adaptor <i>EcoRI-XmnI</i> ^a), <i>Cm</i> ^r	This work
pMS50	pMMB67EHΩ(pVWDG23110 <i>ApaI-BamHI</i> , <i>korA-korB</i> -containing fragment, 5.4 kb), <i>Ap</i> ^r	36
pDB5650	pDB1756Δ1.1Ω(pMS50 <i>ApaI-BamHI</i> , 5.4-kb fragment), <i>Cm</i> ^r	This work
pBS103	pJF118HEΩ(RP4 <i>NotI-BssHII</i> , <i>traFGH</i> -containing fragment, 4.4 kb), <i>Ap</i> ^r	81
pJF119EH	pMB1 replicon, <i>Ptac</i> , <i>Ap</i> ^r	17
pML100	pJF119EHΩ(pBS103 <i>Eco47III-Eco47III</i> , <i>traF</i> , <i>G</i> -containing fragment, 3.1 kb), <i>Ap</i> ^r	This work
pWP471	pJF119EHΩ(RP4 <i>NspHI-HaeII</i> , <i>traF</i> -containing fragment, 0.7 kb), <i>Ap</i> ^r	73
pBS140	pJF119EHΩ(RP4 <i>HincII-SphI</i> , <i>traG</i> -containing fragment, 2.2 kb), <i>Ap</i> ^r	73
RSF1010K ^b	RSF1010 replicon, <i>Km</i> ^r	E. Scherzinger
pMMB67EH	RSF1010 replicon, <i>Ap</i> ^r	17
pMS67EH	RSF1010 repliconΔ <i>oriT</i> , <i>Ap</i> ^r	36
pMS54	pJF119EHΩ(RP4 <i>trbB</i>), <i>Ap</i> ^r	41
pMS80	pMS67EHΩ(RP4 <i>trbB</i>), <i>Ap</i> ^r	36
pML102	pMS67EHΩ(RP4 <i>ScaI-NotI</i> , <i>trbF</i> , <i>G</i> -containing fragment, 2.1 kb), <i>Ap</i> ^r	This work
pMS119EH	pJF119EHΔ(<i>NruI-NdeI</i> , 1.3-kb fragment), <i>Ap</i> ^r	63
pDB21	pMS119EHΩ(pT7-7 <i>XbaI-NdeI</i> , 40-bp fragment, adaptor <i>NdeI-BsaI</i> , ^c pDB1756Δ1.1.4 <i>BsaBI-KpnI</i> , <i>trbE</i> , <i>F</i> -containing fragment, 3.5 kb), <i>Ap</i> ^r	This work
pKW21Δ1 ^d	pMS67EHΩ(pDB21 <i>trbE</i> -containing fragment, 2.7 kb), <i>Ap</i> ^r	This work
pDB179	pMS54Ω(RP4 <i>NdeI-AvaI</i> , partial <i>trbC</i> -containing fragment, 0.4 kb), <i>Ap</i> ^r	This work
pDB379	pMS67EHΩ(pDB179 <i>EcoRI-HindIII</i> , <i>trbB</i> , <i>C</i> -containing fragment, 1.6 kb), <i>Ap</i> ^r	This work
pML20 ^e	pMS67EHΩ(RP4 <i>trbH</i>)	This work
pML40	pJF118EHΩ(RP4 <i>trbJ</i>)	38
pMS172 ^f	pMS67EHΩ(RP4 <i>traM</i>)	This work

^a The nucleotide sequence of the *EcoRI-XmnI* adaptor is shown in Fig. 5.

^b Nucleotide positions 1 through 1340 were derived from Tn903 (positions 878 to 2217 [42]); positions 1341 to 8320 were derived from RSF1010 (positions 1705 to 8684 [56]).

^c The nucleotide sequence of the *NdeI-BsaI*-adaptor used was TATGATCCAAGCAATTGCGATTG
ACTAGGTTCTGTTAACGCGAAC.

^d The construction of pKW21Δ1 will be described elsewhere.

^e The *trbH* gene was cloned with the help of the polymerase chain reaction, in which standard conditions were applied (26). pDB1756Δ1.1.2 was used as a template. The following oligonucleotides were applied as primers: coding strand, 5'-GGCCATCTCATCCCGGCGTGGGAGCAGC (position 6743 in reference 37); noncoding strand, 5'-GCTTGACGCATCTGGGCATGCGTCCGGTGC (position 7345 in reference 37) containing an *SmaI* and an *SphI* restriction site. After amplification, the fragment was digested with *SphI* and *SmaI* and introduced into pMS67EH.

^f For construction of pMS172, *traM* carrying the *EcoRI-HindIII* fragment of pJF172 (81) was inserted into the multiple cloning site of pMS67EH.

of pVWDG23110Δ3, -Δ4, -Δ9, and -Δ10 are similar to those achieved with the complete pVWDG23110 (Table 2). However, the effects of the RP4 primase (*TraC*, pVWDG23110Δ3) in conjugation can be evaluated in this transfer system, because the primase function can be substituted by the host-encoded priming system. In addition, deletion of all of *traC* (pVWDG23110Δ4) shows that the 5' region of this gene, which was proposed to be involved in transport of the DNA strand into the recipient cell (53), is not essential, at least for matings between *E. coli* cells. Furthermore, mating experiments with derivatives pVWDG23110Δ3, -Δ9, and -Δ10 revealed that *TraI* genes A, B, D, and E are not required for the RP4 transfer under the conditions used here.

Deletion from the righthand site of the *TraI* region (with respect to the map shown in Fig. 1), caused a more gradual loss of transfer ability. The lack of *traO* and *traN* in the derivative pVWDG23110Δ1.1 seemingly did not affect the transfer frequency of this plasmid in the presence of *Tra2* functions. However, deletion of *traM* in pVWDG23110Δ1.2 resulted in a 300-fold decrease in transfer efficiency compared with a strain harboring plasmids with the intact *TraI* and *Tra2* regions. Thus, *traM* is not absolutely required for conjugative transfer of plasmid RP4 between *E. coli* cells, but it apparently increases the efficiency of the transfer process. A progressive deletion into *traL* in the derivative pVWDG23110Δ1.3 did not reduce the transfer frequency any further, suggesting that *traL*

might not be needed for the transfer process between *E. coli* cells. To test this, we inserted a MURFI linker into the *DrdI* restriction site of *traL* (pVWDG23110*traL*71). The transfer frequency achieved by HB101 (pVWDG23110*traL*71, pDB1756Δ1.1) was similar to those of pVWDG23110Δ1.2 and -Δ1.3 (Table 2). However, we considered the possibility that insertion of the MURFI linker into *traL* might have affected translation of the *traM* gene product. The stop and start codons of *traL* and *traM* overlap, suggesting that they are translationally coupled (81). Complementation of pVWDG23110*traL*71 with a *traM* plasmid (pMS172 [Table 1]) increased the transfer frequency (Table 2), supporting this hypothesis. Previous experiments have shown that *traK*, *traJ*, and *traI* are essential transfer functions required for the heterologous mobilization of recombinant *oriT* plasmids by plasmid R751 (18, 22, 23). Accordingly, the section of *TraI* encompassing the *tra* genes *F* to *K* is essential for intraspecific *E. coli* matings. The presence of *traM* increases the transfer frequency significantly (Table 2).

Which of the *TraI* functions are part of the mating pair formation system? To define the *TraI* components of the mating pair formation system, we analyzed the ability of *TraI* deletion derivatives to mobilize the IncQ plasmid RSF1010 and to serve as hosts for propagation of donor-specific bacteriophages. Recent experiments demonstrated that both processes require functions encoded by *TraI* as well as by *Tra2* (36). To elucidate which of the *TraI* functions affect these

FIG. 1. Physical and genetic map of the RP4 TraI region and notation of TraI deletion derivatives. In the upper part of the figure, a physical map of plasmid RP4 between kilobase coordinates 38.0 and 60.1 (according to the RP4 map of Lanka et al. [34]) is shown. This region, present on plasmid pVWDG23110 (Table 1), encompasses the entire TraI region, the gene organization of which is depicted in the lower part of the figure. Genes of this section are drawn as bars; arrowheads mark gene starts. A through O indicate *tra* genes, P indicates promoters, and T indicates rho-independent terminators. Terminators are presented as compiled from references 28, 40, 67, and 81. All promoters shown, except that preceding the *korA* gene, have been verified by RNA-polymerase binding (35, 67). Promoters in the *oriT* region (18) and the promoter preceding the primase operon (40) have also been tested in five different gram-negative bacteria (20). The origin of transfer (*oriT*) is drawn as a hatched segment. The map of pVWDG23110 is based on sequence data (36). Beneath the restriction map, various deletion derivatives of pVWDG23110 are shown, in which the shaded bars represent the remaining part of the plasmid. The corresponding transfer phenotypes of these derivatives in *E. coli* $\times$ *E. coli* matings are listed on the righthand side of the figure. + indicates that the respective derivative is transfer proficient, (+) indicates that the transfer frequency is reduced, and - indicates that the derivative is transfer defective. The corresponding transfer data for these derivatives are presented in Table 2.

abilities, we first had to construct a suitable test system, since the Tra2 derivatives can only be maintained stably in the presence of the *korA-korB* control region, which is normally provided by the Tra1 plasmid pVWDG23110 (Fig. 1). To circumvent this problem, we constructed plasmid pDB5650

TABLE 2. Transfer proficiency of Tra1 deletion mutants

Plasmids in the donor strain HB101	Transfer frequency ^a
pVWDG23110, pDB1756Δ1.1	5×10^{-1}
pVWDG23110Δ3, pDB1756Δ1.1	5×10^{-1}
pVWDG23110Δ4, pDB1756Δ1.1	9×10^{-1}
pVWDG23110Δ9, pDB1756Δ1.1	4×10^{-1}
pVWDG23110Δ10, pDB1756Δ1.1	1×10^{-1}
pVWDG23110Δ11, pDB1756Δ1.1	$< 1 \times 10^{-7}$
pVWDG23110Δ1.1, pDB1756Δ1.1	9×10^{-1}
pVWDG23110Δ1.2, pDB1756Δ1.1	3×10^{-3}
pVWDG23110Δ1.3, pDB1756Δ1.1	1×10^{-2}
pVWDG23110 <i>mt</i> raL71, ^b pDB1756Δ1.1	4×10^{-3}
pVWDG23110 <i>mt</i> raL71, pDB1756Δ1.1, pMS172	9×10^{-2}

^a Transfer frequencies are given as the number of transconjugants per donor cell. The recipient used was HB101 Nx^r. Each number given represents the average value of three experiments.

^b pVWDG23110*mtraL*71 designates the MURFI linker insertion mutant into *traL*.

FIG. 2. Physical and genetic map of pDB5650, a Tra2 plasmid of RP4. For construction of pDB5650, a 5.4-kb *Eco*RI-*Hind*III fragment of plasmid pMSS0 (36) encompassing the entire *korA*-*korB* operon [(a)] was inserted into plasmid pDB1756Δ1.1 (Table 1) via the *Pst*I and *Hind*III restriction sites. To connect and regenerate the *Eco*RI and *Pst*I restriction sites, an adaptor was used (coding strand, 5'-GCG GTCCGAG; noncoding strand, 3'-ACGTCGCCAGGCTCTTAA). A genetic map of the regulation region inserted is shown in the lower part of the figure [(a)]. Genes are drawn as open bars; gene starts are marked by arrowheads. Designations are as described in the legend to Fig. 1. The shaded region in the outer circle of the plasmid map indicates the portion of the vector plasmid pGZ119EH (36), and the open circle indicates the RP4 Tra2 region between restriction sites *Ear*I (18.0 kb) and *Pst*I (30.9 kb). *trb* genes of the RP4 Tra2 transfer region are designated by A through O; the *ssb* gene (in brackets) is truncated (30). *cat* marks the chloramphenicol acetyltransferase gene of the vector. The map of pDB5650 is based on sequence data from the following references: Tra2 region, 29, 37, 41, 68; *kfrA*, *korFI*, and *korFII*, 28; *korB*, 31, 64, 65; *incC* and *korA*, 66; *klaC*, 19.

(Fig. 2 and Table 1), which consists of the Tra2 plasmid pDB1756Δ1.1 carrying the *korA-korB* operon in its single *PstI* site.

Previous results indicated that genes arranged adjacent to *oriT* in the relaxase and leader operon (*traH*, *traI*, *traJ*, *traK*) are involved in the site-specific nicking at the *oriT* (18, 48) and hence are part of the DNA transfer and replication system classified by Willetts (77). Therefore, these components are probably not involved in the formation of mating pairs. In addition, our deletion analysis revealed that *traA* to *-E* of *TraI* are not required for RP4 transfer. Thus, the most likely candidates for the Mpf functions of *TraI* were the gene products of *traF* and *traG*. This assumption is supported by the recent finding that mutation of *traF* rendered these plasmids unable to serve as a host for donor-specific phages (73). To confirm these predictions by documentary evidence, we intro-

FIG. 3. RP4 Tra1 functions essential for RSF1010 mobilization and donor-specific phage propagation. In the upper part of the figure, a physical and genetic map of the RP4 Tra1 region between kilobase coordinates 45.3 and 49.7 is drawn. Only restriction sites relevant for the deletion analyses are shown. *tra* genes are indicated as open bars; gene starts are marked by arrowheads. The hatched bars correspond to the RP4 portion still present in the deletion derivatives (vector plasmids are indicated in Table 1). E.o.p. (EOP) is the number of PFUs of donor-specific phages (Pf3, PRR1, PRD1) on strains of SCS1 harboring the Tra1 deletion derivatives in addition to the Tra2 plasmid pDB5650 (Fig. 2) relative to that of an RP4-containing strain. The EOP of SCS1(RP4) is 1. The second column shows the actual mobilization frequency (Mob) of plasmid RSF1010K from strains harboring the Tra1 deletion derivatives and the Tra2 plasmid pDB5650. The mobilization frequency is given as the number of transconjugants per donor cell, obtained by 1-h standard filter matings. The mobilization frequencies obtained in several experiments ranged from 0.8 to 1.2. SCS1 Rif^r served as a recipient.

duced the plasmid pML100 (Fig. 3 and Table 1) into strain SCS1(pDB5650). pML100 carries the coding regions for the gene products *traF* and *traG*. As shown in Fig. 3, the resulting strain SCS1(pDB5650, pML100), was able to mobilize the IncQ plasmid RSF1010 and was sensitive to donor-specific phages. Thereby, the mobilization frequency, as well as the EOP, matches those of RP4-carrying strains, indicating that all essential functions are provided by this system. To answer the question of whether either *traF* or *traG* alone is sufficient for RSF1010 mobilization or the propagation of donor-specific phages, we constructed strains carrying either *traF* (pWP471) or *traG* (pBS140) in *trans* to the Tra2 plasmid pDB5650 (Fig. 3). The results shown identify *traF* as the only Tra1 function necessary to confer host strain sensitivity to donor-specific phages. However, *traG*, in addition to *traF*, is required for the mobilization of RSF1010. Complementation studies of *traG* mutants with pBS140 have demonstrated that this plasmid provides a functional TraG protein (73).

Generation of RP4 Tra2 mutants. Recently, 12 ORFs designated *trbA* to *-L* were identified within the Tra2 core region essential for intraspecific *E. coli* matings (37). For initial functional analyses of the gene products, we generated a series of RP4 Tra2 mutants with the help of a so-called MURFI linker (52). This linker sequence, containing a central *Xba*I restriction site and amber nonsense codons (TAG) in all three reading frames, was inserted into unique restriction sites located in the RP4 Tra2 region (Fig. 4). Each of the resulting Tra2 derivatives was checked by restriction analysis and nucleotide sequencing to verify the MURFI linker insertion. The linker mutants were designated pDB1756*trbX*000. The number indicates the last amino acid of the truncated gene product. The strategy of MURFI linker insertion was chosen to obtain defined mutants and to minimize the severity of transcriptional

TABLE 3. Complementation analyses of the RP4 Tra2 mutants

Plasmids in the donor strain HB101	Transfer frequency ^a
pVWDG23110, pDB1756Δ1.1.3	7×10^{-1}
pVWDG23110, pDB1756 <i>trbB</i> 204 ^b	$<1 \times 10^{-7}$
pVWDG23110, pDB1756 <i>trbB</i> 204, pMS80	2×10^{-2}
pVWDG23110, pDB1756 <i>trbC</i> 45	$<1 \times 10^{-7}$
pVWDG23110, pDB1756 <i>trbC</i> 45, pDB379	1×10^{-2}
pVWDG23110, pDB1756 <i>trbE</i> 402	$<1 \times 10^{-7}$
pVWDG23110, pDB1756 <i>trbE</i> 402, pKW21Δ1	3×10^{-2}
pVWDG23110, pDB1756 <i>trbG</i> 145	$<1 \times 10^{-7}$
pVWDG23110, pDB1756 <i>trbG</i> 145, pML102	6×10^{-3}
pVWDG23110, pDB1756 <i>trbI</i> 365	1×10^{-2}
pVWDG23110, pDB1756 <i>trbL</i> 184	$<1 \times 10^{-7}$

^a Transfer frequencies are expressed as the ratio of the number of transconjugants per donor cell. The recipient used was HB101 Nx'. Each number represents the average value of three experiments.

^b pVWDG23110*trbB*204 to *-trbL*184 designate insertions of the MURFI linker in respective Tra2 ORFs.

polarity (52, 73), which is often observed with conventional transposon mutagenesis (8).

Transfer phenotypes of RP4 Tra2 mutants. To determine the transfer phenotype of each of the Tra2 mutants, the strain HB101(pVWDG23110) was transformed with the respective plasmids. Subsequent mating experiments revealed that the transfer frequencies of the Tra2 derivatives pDB1756*trbB*204, *trbC*45, *trbE*402, *trbG*145 and *trbL*184 were $<1 \times 10^{-7}$ (Table 3 and Fig. 4). In contrast, linker insertion into *TrhIII1* (pDB1756*trbI*365) resulted in a reduced transfer frequency of the Tra1 plasmid pVWDG23110 (Table 3), but did not abolish the ability to transfer. This might suggest that the *trbI* gene product contributes to but is not essential for the transfer process. However, we cannot exclude the possibility that the N-terminal part of the *trbI* gene product is essential for conjugative transfer, since the linker was inserted near the 3' end of the coding region (Fig. 4), producing a 365-amino-acid (aa) polypeptide (98 of a total of 463 aa are missing).

To rule out severe polar effects of the mutations on downstream genes *trans* complementation assays of the transfer-deficient mutants were performed. Respective genes were subcloned into an *oriT*-deficient RSF1010 derivative (pMS67EH [Table 1]) and were introduced in strains harboring pVWDG23110 in addition to the mutant Tra2 plasmids. The RP4 portions used for the complementation analyses are shown in the upper part of Fig. 4. Transfer proficiency of Tra2 mutants in *trbB*, *-C*, *-E*, and *-G* could be restored by complementation with respective plasmids in *trans* (Table 3), confirming that at least severe polar effects did not influence this study. For the *trbI* mutant (pDB1756*trbI*365), no complementation assay was done, because this derivative is transfer proficient. Similarly, it was unnecessary to test for polarity defects by complementation of pDB1756*trbL*184, since *trbL* is the most distal gene in the Tra2 core region (37).

We conclude from our results that the Tra2 gene products *trbB*, *-C*, *-E*, *-G*, and *-L* are essential components of the conjugative RP4 system, whereas at least the C-terminal part of TrbI is not absolutely required for this process.

Functions of Tra2 involved in the formation of mating pairs between conjugating cells. To find out which of these Tra2 gene products are part of the Mpf system, phenotypes of the mutants concerning RSF1010 mobilization and phage propagation were determined. This was done as described for the Tra1 derivatives, with the modification that the Ap^r RSF1010 derivative pMMB67EH was used for the mobilization studies

FIG. 4. Mutational analyses of RP4 Tra2 genes. In the middle of the figure, a physical and genetic map of the RP4 Tra2 region is presented. Restriction sites used for the insertion of MURFI linkers were *AatII*, *NdeI*, *AgeI*, *RsrII*, *Tth111I*, and *BspEI*. Promoters are indicated by P₂ to P₄, *trb* genes are indicated by capital letters, and the gene starts are indicated by arrowheads. The phenotypes of the insertion mutants are listed below. Tra indicates whether the respective Tra2 derivative is able to provide all functions essential for the transfer of the Tra1 plasmid pVWDG23110; (+) stands for a reduced transfer frequency; - means that the transfer frequency is $<1 \times 10^{-7}$ (for precise data, see Table 3). The results of complementation analyses (Compl.) of these mutants are shown. + indicates that the mutant could be complemented by providing the appropriate gene product in *trans* (Table 3). N.D., not determined. The RP4 portions of derivatives used for the complementation analyses are drawn in the upper part of the figure. On the third line, the sensitivity towards donor-specific phages (Pf3, PRR1, PRD1) of host cells carrying respective Tra2 mutants in addition to pVWDG23110 is shown (Dps); - means that no propagation of phages could be observed; (-) means that no plaques could be observed upon infection of the cells with phages PRD1 and PRR1. Pf3 does not form clearly distinguishable plaques but does elicit slight zones of growth inhibition when spotted on surface-inoculated lawns. On the last line, the ability of these mutants to mobilize the RSF1010 derivative pMMB67EH is listed. - shows that the mobilization frequency is $<1 \times 10^{-7}$; (+) indicates a mobilization frequency of 2.4×10^{-2} .

(Table 1). The results of these studies are summarized in Fig. 4. None of the Tra2 derivatives, except pDB1756 Δ *trbI365*, was able to mobilize RSF1010 or to serve as a host for phage propagation. Only the *trbI* mutant, which also exhibits a transfer-proficient phenotype (Table 3), could mobilize RSF1010, although with a reduced mobilization frequency. Propagation of phages was not observed on a strain harboring the mutant *trbI* plasmid. Only infection with Pf3 seemed to result in a slight reduction of host strain growth. Altogether, gene products of at least *trbB*, -C, -E, -G, and -L are involved in establishing the contact between donor and recipient cells during the conjugative process.

Is *trbA* an essential transfer function? Initial studies identified *trbA* (Fig. 4) as a transcriptional regulator (29), repressing transcription of *trfA* (vegetative replication) and *trbB* (conjugative transfer) and, in this way, controlling both vegetative replication and conjugative DNA transfer. In addition to its regulatory role, we had assumed that it is an essential *tra* gene product, because the Tra2 deletion derivative pDB1756 Δ 2.1, containing only the 3' end of *trbA*, expressed a transfer-defective phenotype (36). We wanted to verify this result by site-specific introduction of a MURFI linker into the *trbA* coding region. However, several attempts to obtain such a *trbA*

FIG. 5. Construction of plasmid pML123. The upper part shows a physical and genetic map of the RP4 portion of plasmid pDB1756 Δ 1.1.2. Arrowheads mark gene starts, P₂ to P₄ mark promoters, and O_B and O_A mark binding sites for KorB and KorA, respectively (2, 59, 60). A to C and N mark Tra2 transfer genes. For construction of pML123, a 0.9-kb *EcoRI*-*XmnI* fragment was deleted from pDB1756 Δ 1.1.2, whereby the *trbB* ORF (start codon is indicated by an arrow) and the native ribosome-binding site (SD) were restored by insertion of an appropriate adaptor. The adaptor sequence is shown in lowercase letters in the lower part of the figure.

mutant in pDB1756 Δ 1.1.2 failed. Therefore, we decided to replace the entire regulatory region including the Tra2 promoter(s) with the LacI-regulated *tac* promoter (Fig. 5). To obtain this plasmid, an *EcoRI*-*XmnI* fragment was deleted from pDB1756 Δ 1.1.2, removing *trbA* as well as the entire Tra2 control region. To restore the *trbB* structural gene with its native ribosome-binding site, a synthetic oligonucleotide was used. The resulting plasmid, pML123 (Fig. 5), could be stably maintained in the absence of the *korA-korB* operon, which had been thought to be essential for cloning of the Tra2 region (36).

To determine the transfer phenotype of pML123, we introduced this plasmid into HB101(pVWDG23110). In contrast to our expectations arising from previous results, this strain was transfer proficient, expressing a transfer frequency (9×10^{-1}) identical to that of HB101(pVWDG23110, pDB1756 Δ 1.1), 5×10^{-1} . Since pML123 was independent of the presence of the RP4 key regulatory region, we expected that the RP4 *korA-korB* region is not essential for RP4 transfer. To test this, we transformed a strain harboring pVWDG23110 Δ 0.1 with pML123. pVWDG23110 Δ 0.1 is a Tra1 plasmid lacking the *korA-korB* operon (Fig. 1). As expected, HB101(pVWDG23110 Δ 0.1, pML123) was able to transfer the Tra1 plasmid (9×10^{-1} transconjugants per donor) with a frequency comparable to that of HB101(pVWDG23110, pDB1756 Δ 1.1). Accordingly, these results imply that *trbA* is not an essential transfer gene product. Furthermore, the *korA-korB* control region, encoding the two major regulatory proteins KorA and KorB, no longer seems to be required in *trans* in a derivative lacking *trbA* and the Tra2 promoter region. Thus, these manipulations led to a refined binary plasmid system that can be handled more easily.

Immunological identification of pML123-encoded Tra2 gene products. To prove that the *trbB* reading frame was restored properly and that transcription of the Tra2 region takes place, we monitored expression of TrbB and TrbJ by immunoblotting. Both gene products were detectable in extracts of SCS1(pML123) as well as those of HB101(pVWDG23110, pML123) (Fig. 6). Interestingly, expression of Tra2 genes by transcriptional induction was achieved only in strains harboring pML123. Therefore, induction of pML123 in SCS1 as a host resulted in higher amounts of protein than induction of the plasmid in strain HB101. This is a phenomenon which has

FIG. 6. Identification of RP4 TrbB and TrbJ proteins by solid-phase immunoassay. Extracts of HB101(pVWDG23110, pDB1756Δ1.1), HB101(pVWDG23110, pML123), and SCS1(pML123) were electrophoresed on an SDS-polyacrylamide gel (15% polyacrylamide). +, extracts of IPTG-induced cells; -, uninduced cells. Proteins were identified by anti-TrbB serum (diluted 1:500) and anti-TrbJ serum (diluted 1:750) (preparation of antisera is described elsewhere). The first lane shows the molecular mass standards (fluorescein isothiocyanate-bovine serum albumin [BSA] and fluorescein isothiocyanate-lysozyme [LYS]), the second lane shows extract of HB101, and the third lane shows the antibody reaction with the respective purified protein.

been observed previously in different cases (38a). No overproduction could be observed in strains carrying pDB1756Δ1.1 (Fig. 6), the Tra2 plasmid with the natural Tra2 promoter situation, although even in this plasmid the *tac* promoter is located upstream of the Tra2 region. Identification of TrbJ by immunoblotting revealed that the unprocessed form of the TrbJ protein also exists in extracts of induced pML123-carrying strains (TrbJ [Fig. 6]).

On Coomassie blue-stained polyacrylamide gels, no Tra2 gene products were identifiable (data not shown) in extracts prepared from HB101 (pVWDG23110, pDB1756Δ1.1). The Tra2 proteins could only be detected by radioactive labeling with the T7 promoter system (37) or by immunological cross-reaction with antibodies. However, extracts of IPTG-induced

FIG. 7. Visualization of Tra2 proteins. Extracts of SCS1 cells carrying the indicated plasmids were loaded onto an SDS-polyacrylamide gel (15% polyacrylamide). +, extracts of IPTG-induced cells; -, extracts of uninduced cells. Tra2 gene products are listed on the lefthand side of the figure. An asterisk indicates the processed form of these gene products. In cases in which no clear assignment of predicted Tra2 proteins was possible, only the calculated molecular masses of newly appearing bands in the induced extract of SCS1(pML123) were given. Standard proteins are aprotinin (APRO; 6.8 kDa), lysozyme (LYS; 14.3 kDa), chymotrypsin A (CHYA; 25.7 kDa), ovalbumin (OVA; 43.6 kDa), and bovine serum albumin (BSA; 68 kDa).

SCS1(pML123) cells analyzed by SDS-polyacrylamide gel electrophoresis included a set of additional bands that were absent in uninduced cells (Fig. 7), indicating that this derivative allows production of Tra2 gene products in large amounts. To identify at least some of the proteins visible in induced extracts of SCS1(pML123), we compared the obtained band pattern with those of several overproduced Tra2 proteins (Fig. 7). Bands corresponding to TrbB, -E, -F, -G, and -J were clearly visible in the extract of SCS1(pML123). A band matching the overproduced TrbH protein of pML20 could not be seen in the pML123 pattern, indicating a lower level of overproduction. In cases in which no clear correlation of gene products with respective bands was possible, only calculated molecular masses are given (Fig. 7).

DISCUSSION

traF, -G, -I, -J, and -K are sufficient for RP4 transfer, if Tra2 functions are provided in *trans*. The deletion analyses defined the Tra1 core region as a segment ranging from genes *traF* to -K (Fig. 1 and Table 2). Accordingly, *traA* to -E, as well as *traL* to -O, are not absolutely required for transfer between *E. coli* cells. Supply of *traM*, however, increases the transfer efficiency (Table 2), suggesting that the gene product might be important for conjugation under suboptimal conditions. It is also conceivable that the genes *traA* to -E and *traL* to -O are required for efficient plasmid transfer from or to bacterial species different from *E. coli*. This has been examined for the *traC* gene encoding the RP4-specified primase. Mutation of *traC* led to

significant reduction of plasmid transfer to certain species, such as *Proteus mirabilis* or *Salmonella typhimurium* (33).

Our deletion analysis identified *traF* as an essential transfer gene. This confirms the data of Waters et al. (73) demonstrating that insertion of a MURFI linker in *traF* caused a loss of transfer ability. The same approach was applied to *traG*, revealing that *traG* is also required for IncP conjugation. Mutation of *traH*, however, resulted in a transfer-proficient derivative. This indicates that *traH*, which stabilizes relaxosomes in vitro (48), is not an essential RP4 transfer gene (11). Since the *traI* gene product bears the site- and strand-specific cleaving-joining activity at the *oriT* (48, 50), it is clearly essential for conjugative transfer. Gene products of *traJ* and *traK*, both of which bind specifically to the nick region of the *oriT* (79, 81), were shown to be essential for heterologous mobilization of RP4 recombinant *oriT* plasmids in *trans* by R751 (18). Furthermore, their requirement for conjugative transfer was shown by Tn5 mutagenesis (22). Hence, we conclude that only six loci of the Tra1 region, namely *traF*, *-G*, *-I*, *-J*, and *-K*, including *oriT*, are required for RP4 transfer between *E. coli* cells. Accordingly, the Tra1 genes *traA* to *-E* and *traL* to *-O* as well as the Tra2 genes *trbM* to *-P* (37) are not required for intraspecific *E. coli* matings under the conditions applied. This might be due to one of several reasons. (i) The genes mentioned above might be complemented by host-encoded proteins. (ii) They might play an accessory role in other than *E. coli* matings. (iii) They are not transfer genes.

Tra1 functions required for mobilization of plasmid RSF1010 are *traF* and *-G*, whereas *traF* alone is sufficient for donor-specific phage growth. Former investigations indicated that functions in both transfer regions, Tra1 and Tra2, are necessary for IncQ mobilization (36) and phage propagation (4, 13, 62, 74). Our complementation and deletion analysis has identified *traF* and *traG* as the only Tra1 components required in *trans* with Tra2, for expression of a Mob⁺ phenotype, whereas for phage growth, *traF* in addition to *trb* genes is sufficient. A similar investigation of the RP4 Tra1 region was done recently by Fong and Stanisich (15). In contrast to our results, they assumed that *traF* and at least a part of *traG* are essential for propagation of phages PR4 and PRR1. However, they did not sequence their Tra1 mutations, so that a clear assignment to Tra1 ORFs cannot be made.

Recently, Beijersbergen et al. (7) demonstrated that VirD4, together with the VirB functions normally involved in T-DNA transfer to plant cells, can direct conjugative transfer of RSF1010 between agrobacteria. This parallels our data regarding the RSF1010 mobilization studies, because extensive sequence similarities were found between TraG and VirD4 (81) as well as between several VirB and the Tra2 proteins (37, 41). However, an analog for the RP4 *traF* gene has not yet been found in the Ti system. Considering the low transfer frequencies obtained for RSF1010 transfer between agrobacteria (approximately 2×10^{-5} [7]) with respect to the high ones obtained with IncP plasmids (5, 78), we might speculate that a *traF*-like protein would increase transfer efficiency. It is conceivable that *traF* somehow improves the cell-to-cell contact between the donor and the recipient cell, perhaps by promoting formation or assembly of the conjugative pilus. The hydrophobic character of the protein in addition to its potential signal sequence at the N terminus (73) would permit such a role in conjugative transfer.

However, TraG is not only similar to VirD4, but it is also similar to TraD of plasmid F (39). F TraD is, like TraG, an essential *tra* gene product (27), which is probably located in the cytoplasmic membrane (47). Since VirD4 was detected in the cytoplasmic membrane fraction (43), we propose a similar

location for TraG. This assumption is supported by the hydrophobicity of the protein as well as by a potential signal sequence for protein export at its N terminus (81). TraG is not involved in the formation of relaxosomes (18, 48). It is also unlikely that TraG is needed for the formation of the cell-to-cell contact because the *traG* gene is not essential for phage growth (Fig. 2). Therefore, we suggest that TraG might connect the relaxosome with proteins enabling the membrane passage of the DNA. This hypothesis is underscored by the arrangement of TraG downstream of genes specifying the DNA relaxase (*traJ* and *traI* [81]). However, we cannot exclude that TraG is a component of the mating pair complex, which is not required for phage infection.

Summarizing our results, we now might separate the Tra1 gene products into three functional groups: (i) functions involved in conjugative DNA processing (*traI*, *-J*, *-K*), (ii) gene products belonging to the mating pair formation system (*traF*), and (iii) functions that can be grouped to neither i nor ii and that potentially connect both systems (*traG*).

What might be the role of *trbA* during conjugative DNA transfer? Recent experiments have indicated that *trbA* might be a DNA-binding protein which represses transcription of the *trfA* operon as well as the Tra2 region (29). Since these results implied a regulatory instead of a structural role for *trbA* in the transfer process, we reexamined the contribution of *trbA* in conjugative transfer. An essential prerequisite for this project was the availability of the nucleotide sequence (29, 41) of this region, which permitted us to generate defined *trbA* mutations while keeping the rest of Tra2 intact. Thus, we constructed plasmid pML123 by replacing the Tra2 promoter region including *trbA* by a LacI-regulated *tac* promoter. This derivative was transfer proficient when tested in the binary plasmid system, indicating that *trbA* is dispensable. Hence, our results confirm the prediction of Jagura-Burdzy et al. (29) that *trbA* is likely to play a role in RP4 gene expression of the *trfA* and the Tra2 operons.

These findings seem to contrast with previous results obtained with plasmid pDB1756Δ2.1, a Tra2 derivative with a defective *trbA* gene but an intact promoter region upstream of *trbB*. Transfer tests with this plasmid in the binary plasmid system exhibited a transfer-defective phenotype (36). Since pDB1756Δ1.1, a Tra2 plasmid carrying *trbA* in addition to the intact regulatory region, was transfer proficient (36), it is conceivable that TrbA might contribute to stabilizing this plasmid by binding to the control region upstream of *trbB*. It might limit the degree of Tra2 gene expression. Indeed, we found that pDB1756Δ1.1 seems to be maintained more stably in the cell after storage in glycerol cultures at -70°C than pDB1756Δ2.1, even if *korA-korB* functions are provided in *trans*. Moreover, we as well as other laboratories (68) were not able to obtain a *trbA* mutant plasmid, on which the *trbA* ORF was destroyed but the Tra2 promoter region was left intact. In plasmid pML123, however, this effect seems to be overcome, since the potential targets for the proposed TrbA interaction have been deleted and replaced by a unidirectional promoter. Undoubtedly a series of further experiments are needed to elucidate the role of TrbA in gene regulation of conjugative transfer and vegetative plasmid replication.

Tra2 is transcribed as one transcriptional unit. Previous data from RNA-polymerase binding studies and a computer search for σ^{70} consensus sequences indicated that the entire Tra2 region most probably is transcribed from two promoters located upstream of *trbB*. The position of P₄ (Fig. 4) was recently confirmed by primer extension analyses (68). The overexpression of a series of Tra2 proteins, achieved by IPTG induction of the *tac* promoter in pML123 (Fig. 5), is consistent

FIG. 8. Alignment of the RP4 Tra2 (37), the Ti VirB (70, 71), and the Ptl operon of *B. pertussis* (75). A through L indicate ORFs in the RP4 Tra2 region, whereas Ti VirB and *B. pertussis* Ptl ORFs are designated by numbers. Related ORFs are shown by identical shading.

with the existence of a long polycistronic transfer operon (68). Since induction of HB101(pVWDG23110, pDB1756Δ1.1) did not result in obvious Tra2 protein overproduction, expression of Tra2 genes seems to be strictly regulated. According to these results, regulation of Tra2 transcription is subject to the region between *trbB* and *ssb*, as proposed by other groups (29, 68).

***trbB*, -C, -E, -G, and -L encode for essential RP4 transfer genes belonging to the mating pair formation system.** Our mutational analysis of Tra2 genes revealed that five of the six genes tested, namely *trbB*, -C, -E, -G, and -L, are absolutely required for RP4 transfer, RSF1010 mobilization, and donor-specific phage growth. Hence, all of these proteins can be classified as mating pair functions. In addition, recent studies of Kotilainen et al. (32) indicated that the receptor for phage PRD1 is a highly expressed cell surface complex which is responsible for both PRD1 adsorption and conjugation ability.

Moreover, the necessity of *trbB* for IncP conjugative transfer was shown previously (41, 68). The only Tra2 mutant expressing a Tra⁺ phenotype was pDB1756m*trbI*365 (Table 3). Since the MURFI-linker is inserted near the 3' end of the gene (Fig. 4), we conclude that the 98 aa at the C terminus of TrbI (463 aa) are not absolutely required for transfer. Whether the residual part of the protein is essential for conjugation remains to be tested by construction of an additional *trbI* mutant plasmid. As noted, some of the Tra2 proteins exhibit sequences with similarities to the sequences of the VirB proteins of the *A. tumefaciens* Ti plasmid (TrbB-VirB11, TrbC-VirB2, TrbE-VirB4, TrbF-VirB5, TrbI-VirB10 [37, 41]). Interestingly, two additional operons, namely, the Pilw region of the IncW plasmid R388 (11a, 57) and the Ptl region of *Bordetella pertussis* (57, 75), were found to be related to the RP4 Tra2 region (Fig. 8). Gene products of the Pilw region are functionally closely related to the Tra2 proteins, since they also encode *tra* genes most likely involved in the formation of mating pairs (9). The Ptl proteins, however, are chromosomally encoded and most probably are not involved in a DNA transport process. They are proposed to form an export system required for excretion of the pertussis toxin (75). Accordingly, we might assume that gene products of the operons mentioned above specify a more general transport system, which may have evolved from a common ancestor.

ACKNOWLEDGMENTS

We are indebted to Heinz Schuster for generous support and stimulating discussions. We thank David Figurski for providing data prior to publication and critical reading of the manuscript. Marianne Schlicht is appreciated for expert technical assistance and Werner Schröder is appreciated for synthesizing oligonucleotides.

This work was supported by the Sonderforschungsbereich grant 344/B2 of the Deutsche Forschungsgemeinschaft to E.L.

REFERENCES

1. Ayres, E. K., S. Saadi, H. C. Schreiner, V. J. Thomson, and D. H. Figurski. 1991. Differentiation of lethal and nonlethal, *kor*-regulated functions in the *kilB* region of broad host-range plasmid RK2. *Plasmid* 25:53-63.
2. Balzer, D., G. Ziegelin, W. Pansegrau, V. Kruff, and E. Lanka. 1992. KorB protein of promiscuous plasmid RP4 recognizes inverted sequence repetitions in regions essential for conjugative plasmid transfer. *Nucleic Acids Res.* 20:1851-1858.
3. Barth, P. T. 1979. RP4 and R300B as wide host-range plasmid cloning vehicles, p. 399-410. In K. N. Timmis and A. Pühler (ed.), *Plasmids of medical, environmental and commercial importance*. Elsevier/North-Holland Biomedical Press, Amsterdam.
4. Barth, P. T., N. J. Grinter, and D. E. Bradley. 1978. Conjugal transfer system of plasmid RP4: analysis by transposon 7 insertion. *J. Bacteriol.* 133:43-52.
5. Barth, P. T., L. Tobin, and G. S. Sharpe. 1981. Development of broad-host-range plasmid vectors, p. 433-448. In S. B. Levy, R. C. Clowes, and E. L. Koenig (ed.), *Molecular biology, pathogenicity and ecology of bacterial plasmids*. Plenum, New York.
6. Bechhofer, D. A., J. A. Kornacki, W. Firshein, and D. H. Figurski. 1986. Gene control in broad host range plasmid RK2: expression, polypeptide product and multiple regulatory functions of *korB*. *Proc. Natl. Acad. Sci. USA* 83:394-398.
7. Beijersbergen, A., A. K. den Dulk-Ras, R. A. Schilperoort, and P. J. J. Hooykaas. 1992. Conjugative transfer by the virulence system of *Agrobacterium tumefaciens*. *Science* 256:1324-1327.
8. Berg, C. M., D. E. Berg, and E. A. Groisman. 1989. Transposable elements and the genetic engineering of bacteria, p. 879-925. In D. E. Berg and M. M. Howe (ed.), *Mobile DNA*. American Society for Microbiology, Washington, D.C.
9. Bolland, S., M. Llosa, P. Avila, and F. de la Cruz. 1990. General organization of the conjugal transfer genes of the IncW plasmid R388 and interactions between R388 and IncN and IncP plasmids. *J. Bacteriol.* 172:5795-5802.
10. Boyer, H. W., and D. Roulland-Dussoix. 1969. A complementation analysis of the restriction and modification of DNA in *Escherichia coli*. *J. Mol. Biol.* 41:459-472.
11. Cole, S. P., E. Lanka, and D. Guiney. 1993. Site-directed mutations in the relaxase operon of RP4. *J. Bacteriol.* 175:4911-4916.
- 11a. de la Cruz, F. Personal communication.
12. Engström, P., P. Zambryski, M. van Montague, and S. Stachel. 1987. Characterization of *Agrobacterium tumefaciens* virulence proteins induced by the plant factor acetosyringone. *J. Mol. Biol.* 197:635-645.
13. Figurski, D., R. Meyer, D. S. Miller, and D. R. Helinski. 1976. Generation *in vitro* of deletions in the broad host range plasmid RK2 using phage Mu insertions and a restriction endonuclease. *Gene* 1:107-119.
14. Figurski, D. H., R. F. Pohlman, D. H. Bechhofer, A. S. Prince, and C. A. Kelton. 1982. Broad host range plasmid RK2 encodes multiple *kil* genes potentially lethal to *Escherichia coli* host cells. *Proc. Natl. Acad. Sci. USA* 79:1935-1939.
15. Fong, S. T., and V. A. Stanisich. 1993. Identification and characterization of RP1 Tra1 cistrons involved in pilus function and plasmid mobilization. *J. Bacteriol.* 175:448-456.
16. Frey, J., and M. Bagdasarian. 1989. The molecular biology of IncQ plasmids, p. 79-94. In C. M. Thomas (ed.), *Promiscuous plasmids of Gram-negative bacteria*. Academic Press, Inc., Ltd., London.
17. Fürste, J. P., W. Pansegrau, R. Frank, H. Blöcker, P. Scholz, M. Bagdasarian, and E. Lanka. 1986. Molecular cloning of the RP4 primase region in a multi-host-range *tacP* expression vector. *Gene* 48:119-131.
18. Fürste, J. P., W. Pansegrau, G. Ziegelin, M. Kröger, and E. Lanka. 1989. Conjugative transfer of promiscuous IncP plasmids: interaction of plasmid-encoded products with the transfer origin. *Proc. Natl. Acad. Sci. USA* 86:1771-1775.
19. Goncharoff, P., S. Saadi, C.-H. Chang, L. H. Saltman, and D. H. Figurski. 1991. Structural, molecular, and genetic analysis of the *kilA* operon of broad-host-range plasmid RK2. *J. Bacteriol.* 173:3463-3477.
20. Greener, A., S. M. Lehman, and D. R. Helinski. 1992. Promoters

- of the broad host range plasmid RK2: analysis of transcription (initiation) in five species of Gram-negative bacteria. *Genetics* **130**:27–36.
21. Guiney, D. G. 1993. Broad host-range conjugation and mobilizable plasmids in gram-negative bacteria, p. 75–97. In D. B. Clewell (ed.), *Bacterial conjugation*. Plenum Publishing, New York.
 22. Guiney, D. G., C. Deiss, V. Simnad, L. Yee, W. Pansegrau, and E. Lanka. 1989. Mutagenesis of the Tra1 core region of RK2 by using Tn5: identification of plasmid-specific transfer genes. *J. Bacteriol.* **171**:4100–4103.
 23. Guiney, D. G., and E. Lanka. 1989. Conjugative transfer of IncP plasmids, p. 27–56. In C. M. Thomas (ed.), *Promiscuous plasmids of Gram-negative bacteria*. Academic Press, Inc., Ltd., London.
 24. Hanahan, D. 1983. Studies on transformation of *Escherichia coli* with plasmids. *J. Mol. Biol.* **166**:557–580.
 25. Heinemann, J. A., and G. F. Sprague, Jr. 1989. Bacterial conjugative plasmids mobilize DNA transfer between bacteria and yeast. *Nature (London)* **340**:205–209.
 26. Innis, M. A., D. H. Gelfand, J. J. Sninsky, and T. J. White. 1990. PCR protocols. A guide to methods and applications, p. 1–12. Academic Press, New York.
 27. Ippen-Ihler, K. A., and E. G. Minkley. 1986. The conjugation system of F, the fertility factor of *Escherichia coli*. *Annu. Rev. Genet.* **20**:593–624.
 28. Jagura-Burdzy, G., J. P. Ibbotson, and C. M. Thomas. 1991. The *korF* region of broad-host-range plasmid RK2 encodes two polypeptides with transcriptional repressor activity. *J. Bacteriol.* **173**:826–833.
 29. Jagura-Burdzy, G., F. Khanim, C. A. Smith, and C. M. Thomas. 1992. Crosstalk between plasmid vegetative replication and conjugative transfer: repression of the *trfA* operon by *trbA* of broad host range plasmid RK2. *Nucleic Acids Res.* **20**:3939–3944.
 30. Jovanovic, O. S., E. K. Ayres, and D. H. Figurski. 1992. The replication initiator operon of promiscuous plasmid RK2 encodes a gene that complements an *Escherichia coli* mutant defective in single-stranded DNA-binding protein. *J. Bacteriol.* **174**:4842–4846.
 31. Kornacki, J. A., P. J. Balderes, and D. H. Figurski. 1987. Nucleotide sequence of *korB*, a replication control gene of broad host-range plasmid RK2. *J. Mol. Biol.* **198**:211–222.
 32. Kotilainen, M. M., A. M. Grahn, J. K. H. Bamford, and D. H. Bamford. 1993. Binding of an *Escherichia coli* double-stranded DNA virus PRD1 to a receptor coded by an IncP-type plasmid. *J. Bacteriol.* **175**:3089–3095.
 33. Lanka, E., and P. T. Barth. 1981. Plasmid RP4 specifies a deoxyribonucleic acid primase involved in its conjugal transfer and maintenance. *J. Bacteriol.* **148**:769–781.
 34. Lanka, E., R. Lurz, and J. P. Fürste. 1983. Molecular cloning and mapping of the *SphI* restriction fragments of plasmid RP4. *Plasmid* **10**:303–307.
 35. Lanka, E., R. Lurz, M. Kröger, and J. P. Fürste. 1984. Plasmid RP4 encodes two forms of a DNA primase. *Mol. Gen. Genet.* **194**:65–72.
 36. Lessl, M., D. Balzer, R. Lurz, V. L. Waters, D. G. Guiney, and E. Lanka. 1992. Dissection of IncP conjugative plasmid transfer: definition of the transfer region Tra2 by mobilization of the Tra1 region in *trans*. *J. Bacteriol.* **174**:2493–2500.
 37. Lessl, M., D. Balzer, W. Pansegrau, and E. Lanka. 1992. Sequence similarities between the RP4 Tra2 and the Ti VirB region strongly support the conjugation model for T-DNA transfer. *J. Biol. Chem.* **267**:20471–20480.
 38. Lessl, M., V. Krishnapillai, and W. Schilf. 1991. Identification and characterization of two entry exclusion genes of the promiscuous IncP plasmid R18. *Mol. Gen. Genet.* **227**:120–126.
 - 38a. Lessl, M., and E. Lanka. Unpublished observations.
 39. Lessl, M., W. Pansegrau, and E. Lanka. 1992. Relationship of DNA-transfer systems: essential transfer factors of plasmids RP4, Ti and F share common sequences. *Nucleic Acids Res.* **20**:6099–6100.
 40. Miele, L., B. Strack, V. Kruff, and E. Lanka. 1991. Gene organization and nucleotide sequence of the primase region of IncP plasmids RP4 and R751. *DNA Seq. Map.* **2**:145–162.
 41. Motallebi-Veshareh, M., D. Balzer, E. Lanka, G. Jagura-Burdzy, and C. M. Thomas. 1992. Conjugative transfer functions of broad host range plasmid RK2 are coregulated with vegetative replication. *Mol. Microbiol.* **6**:907–920.
 42. Oka, A., H. Sugisaki, and M. Takanami. 1981. Nucleotide sequence of the kanamycin resistance transposon Tn903. *J. Mol. Biol.* **147**:217–226.
 43. Okamoto, S., A. Toyoda-Yamamoto, K. Ito, I. Takebe, and Y. Machida. 1991. Localization and orientation of the VirD4 protein of *Agrobacterium tumefaciens* in the cell membrane. *Mol. Gen. Genet.* **228**:24–32.
 44. Olsen, R. H., S. June-Sang, and R. H. Gray. 1974. Characteristics of PRD1, a plasmid-dependent broad host range DNA bacteriophage. *J. Virol.* **14**:689–699.
 45. Olsen, R. H., and P. Shipley. 1973. Host range and properties of the *Pseudomonas aeruginosa* R factor R1822. *J. Bacteriol.* **113**:772–780.
 46. Olsen, R. H., and D. D. Thomas. 1973. Characteristics and purification of PRR1, an RNA phage specific for the broad host range *Pseudomonas* R1822 drug resistance plasmid. *J. Virol.* **12**:1560–1567.
 47. Panicker, M. M., and E. G. Minkley. 1992. Purification and properties of the F sex factor TraD protein, an inner membrane conjugal transfer protein. *J. Biol. Chem.* **267**:12761–12766.
 48. Pansegrau, W., D. Balzer, V. Kruff, R. Lurz, and E. Lanka. 1990. *In vitro* assembly of relaxosomes at the transfer origin of plasmid RP4. *Proc. Natl. Acad. Sci. USA* **87**:6555–6559.
 49. Pansegrau, W., and E. Lanka. 1991. Common sequence motifs in DNA relaxases and nick regions from a variety of DNA transfer systems. *Nucleic Acids Res.* **19**:3455.
 50. Pansegrau, W., W. Schröder, and E. Lanka. 1993. Relaxase (TraI) of conjugative plasmid RP4 catalyzes a site- and strand-specific cleaving-joining reaction of single-stranded DNA. *Proc. Natl. Acad. Sci. USA* **90**:2925–2929.
 51. Pansegrau, W., G. Ziegelin, and E. Lanka. 1990. Covalent association of the *traI* gene product of plasmid RP4 with the 5'-terminal nucleotide at the relaxation site. *J. Biol. Chem.* **265**:10637–10644.
 52. Perlman, D., and H. O. Halvorson. 1986. The MURFI linker for multiple reading frame insertion of a sense and nonsense codon into DNA. *Nucleic Acids Res.* **14**:2139–2155.
 53. Rees, C. E. D., and B. M. Wilkins. 1990. Protein transfer into the recipient cell during bacterial conjugation: studies with F and RP4. *Mol. Microbiol.* **4**:1199–1205.
 54. Rohrer, J., and D. E. Rawlings. 1992. Sequence analysis and characterization of the mobilization region of a broad-host-range plasmid, pTF-FC2, isolated from *Thiobacillus ferrooxidans*. *J. Bacteriol.* **174**:6230–6237.
 55. Sambrook, J., E. F. Fritsch, and T. Maniatis. 1989. *Molecular cloning: a laboratory manual*, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
 56. Scholz, P., V. Haring, B. Wittmann-Liebold, K. Ashman, M. Bagdasarian, and E. Scherzinger. 1989. Complete nucleotide sequence and gene organization of the broad-host-range plasmid RSF1010. *Gene* **75**:271–288.
 57. Shirasu, K., and C. I. Kado. 1993. The *virB* operon of the *Agrobacterium tumefaciens* virulence regulon has sequence similarities to B, C and D open reading frames downstream of the pertussis toxin-operon and to the DNA transfer-operons of broad-host range conjugative plasmids. *Nucleic Acids Res.* **21**:353–354.
 58. Simon, R., U. Priefer, and A. Pühler. 1983. A broad host range mobilization system for *in vivo* genetic engineering: transposon mutagenesis in Gram-negative bacteria. *Bio/Technology* **1**:784–791.
 59. Smith, C. A., V. Shingler, and C. M. Thomas. 1984. The *trfA* and *trfB* promoter regions of broad host range plasmid RK2 share common potential regulatory sequences. *Nucleic Acids Res.* **12**:3619–3630.
 60. Smith, C. A., and C. M. Thomas. 1984. Nucleotide sequence of the *trfA* gene of broad host-range plasmid RK2. *J. Mol. Biol.* **175**:251–262.
 61. Stanisich, V. A. 1974. The properties and host range of male-specific bacteriophages of *Pseudomonas aeruginosa*. *J. Gen. Microbiol.* **84**:332–342.
 62. Stokes, H. W., R. J. Moore, and V. Krishnapillai. 1981. Comple-

- mentation analysis in *Pseudomonas aeruginosa* of the transfer genes of the wide host range R plasmid R18. *Plasmid* **5**:202–212.
63. **Strack, B., M. Lessl, R. Calendar, and E. Lanka.** 1992. A common sequence motif, -E-G-Y-A-T-A-, identified within the primase domains of plasmid-encoded I- and P-type DNA primases and the α protein of the *Escherichia coli* satellite phage P4. *J. Biol. Chem.* **267**:13062–13072.
64. **Theophilus, B. D. M., and C. M. Thomas.** 1987. Nucleotide sequence of the transcriptional repressor gene *korB* which plays a key role in regulation of the copy number of broad host range plasmid RK2. *Nucleic Acids Res.* **15**:7442–7450.
65. **Thomas, C. M., and D. R. Helinski.** 1989. Vegetative replication and stable inheritance of IncP plasmids, p. 1–25. *In* C. M. Thomas (ed.), *Promiscuous plasmids of Gram-negative bacteria*. Academic Press, Inc., Ltd., London.
66. **Thomas, C. M., and C. A. Smith.** 1986. The *trfB* region of broad host range plasmid RK2: the nucleotide sequence reveals *incC* and key regulatory gene *trfB/korA/korD* as overlapping genes. *Nucleic Acids Res.* **14**:4453–4469.
67. **Thomas, C. M., B. D. M. Theophilus, L. Johnston, G. Jagura-Burdzy, W. Schilf, R. Lurz, and E. Lanka.** 1990. Identification of a seventh operon on plasmid RK2 regulated by the *korA* gene product. *Gene* **89**:29–35.
68. **Thomson, V. J., O. S. Jovanovic, R. F. Pohlman, C.-H. Chang, and D. H. Figurski.** 1993. Structure, function, and regulation of the *kilB* locus of promiscuous plasmid RK2. *J. Bacteriol.* **175**:2423–2435.
69. **Towbin, H., T. Staehelin, and J. Gordon.** 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc. Natl. Acad. Sci. USA* **76**:4350–4354.
70. **Ward, J. E., D. E. Akiyoshi, D. Regier, A. Datta, M. P. Gordon, and E. W. Nester.** 1988. Characterization of the *virB* operon from an *Agrobacterium tumefaciens* Ti plasmid. *J. Biol. Chem.* **263**:5804–5814.
71. **Ward, J. E., D. E. Akiyoshi, D. Regier, A. Datta, M. P. Gordon, and E. W. Nester.** 1990. Characterization of the *virB* operon from an *Agrobacterium tumefaciens* Ti plasmid. *J. Biol. Chem.* **265**:4768.
72. **Waters, V. L., K. H. Hirata, W. Pansegrau, E. Lanka, and D. G. Guiney.** 1991. Sequence identity in the nick regions of IncP plasmids transfer origins and T-DNA borders of *Agrobacterium* Ti plasmids. *Proc. Natl. Acad. Sci. USA* **88**:1456–1460.
73. **Waters, V. L., B. Strack, W. Pansegrau, E. Lanka, and D. G. Guiney.** 1992. Mutational analysis of essential IncP α plasmid transfer genes *traF* and *traG* and involvement of *traF* in phage sensitivity. *J. Bacteriol.* **174**:6666–6673.
74. **Watson, J., L. Schmidt, and N. Willetts.** 1980. Cloning the *TraI* region of RP1. *Plasmid* **4**:175–183.
75. **Weiss, A. A., F. D. Johnson, and D. L. Burns.** 1993. Molecular characterization of an operon required for pertussis toxin secretion. *Proc. Natl. Acad. Sci. USA* **90**:2970–2974.
76. **Wilkins, B. M., and E. Lanka.** 1993. DNA processing and replication during plasmid transfer between Gram-negative bacteria, p. 105–136. *In* D. B. Clewell (ed.), *Bacterial conjugation*. Plenum Publishing Corp., New York.
77. **Willetts, N.** 1981. Sites and systems for conjugal DNA transfer in bacteria, p. 207–215. *In* S. B. Levy, R. C. Clowes, and E. L. König (ed.), *Molecular biology, pathogenicity, and ecology of bacterial plasmids*. Plenum Press, New York.
78. **Willetts, N., and C. Crowther.** 1981. Mobilization of the non-conjugative IncQ plasmid RSF1010. *Genet. Res.* **37**:311–316.
79. **Ziegelin, G., J. P. Fürste, and E. Lanka.** 1989. *TraI* protein of plasmid RP4 binds to a 19-base pair invert sequence repetition within the transfer origin. *J. Biol. Chem.* **264**:11989–11994.
80. **Ziegelin, G., W. Pansegrau, R. Lurz, and E. Lanka.** 1992. *TraK* protein of conjugative plasmid RP4 forms a specialized nucleoprotein complex with the transfer origin. *J. Biol. Chem.* **267**:17279–17286.
81. **Ziegelin, G., W. Pansegrau, B. Strack, D. Balzer, M. Kröger, V. Kruft, and E. Lanka.** 1991. Nucleotide sequence and organization of genes flanking the transfer origin of promiscuous plasmid RP4. *DNA Seq. Map* **1**:303–327.