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Conjugal Transfer System of the IncN Plasmid pKM101

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The conjugal transfer system of the broad-host range IncN plasmid pKM101 was analyzed genetically. Its organization differed significantly from that of the F plasmid. The *tra* genes are located in three regions, each between 3 and 4 kilobases in length. All of the genes in the first two regions are required for sensitivity to "donor-specific" phage which bind to the plasmid-mediated sex pilus, and these genes therefore are involved in the synthesis, and possibly retraction, of the sex pilus. The plasmid's origin of tranfer was localized to a 1.2-kilobase region at an extreme end of the transfer region. Using two different methods, we have identified 11 complementation groups required for transfer. One of these, *traC*, is of special interest in that mutations at this locus can be partially suppressed if, prior to mating, cells carrying a *traC* mutant plasmid are incubated with cells which elaborate sex pili but are unable to transfer their plasmids. One possible explanation for this is that pilus-elaborating cells can donate *traC* gene product to a *traC* mutant in a form that can be reused.

Conjugation is the process whereby DNA is transferred from one bacterial cell to another by a mechanism requiring cell-to-cell contact. The only conjugal transfer system to have been investigated extensively is that of the F plasmid and its close relatives (9, 20, 31, 32). F contains at least 20 genes which are necessary for transfer, 18 of which are expressed as one transcript about 33 kilobases (kb) in length (14). At least 12 of these cistrons are necessary for pilus formation, and these genes are clustered in one group at the promoter-proximal end of the *tra* operon (32). *traJ* positively controls the transcription of the other 19 genes as well as of genes necessary for entry exclusion (30). Transfer is initiated at a site designated *oriT*, which is localized at one extreme of the transfer region (28).

In contrast, the conjugal transfer systems of broad-host range plasmids such as those of the IncN and IncP groups have been less well characterized. Tn*7* mutagenesis of the IncP plasmid RP4 yielded 19 Tra$^-$ mutants falling into five complementation groups (2) and 4 entry exclusion-deficient mutants, all of which were transfer deficient (1). These 23 Tra$^-$ insertions were physically mapped to three regions of the plasmid.

The conjugal transfer systems of IncF, IncI, IncN, and IncP plasmids seem quite different from each other (29). No plasmid in one group can complement mutations in a different group, and little if any DNA homology has been observed between plasmids of these groups (13). However, plasmids of all four groups code for (i) sex pili, (ii) a special system for conjugal replication and transfer of plasmid DNA, and (iii) products mediating "surface exclusion," which prevent the cell from being used as a recipient by another donor cell carrying the same plasmid. The sex pili of these and other plasmids fall into three categories: thick flexible (IncF), thin flexible (IncI), and thin rigid (IncN and IncP) (4). Bacteriophage have been isolated which specifically attack cells containing plasmids of any one of the four groups but not the other three (16, 29). In addition, phage have been isolated which infect cells containing IncN, IncP, or IncW plasmids but not IncF or IncI plasmids (5). Phage-resistant derivatives of IncF, IncN, and IncP plasmids have been isolated, and these are invariably transfer deficient (2, 8, 10), indicating that these structures are required for conjugation.

In the present study, the conjugal transfer system of the IncN plasmid pKM101 has been investigated. pKM101 was derived from its clinically isolated parent R46 (24) by an in vivo deletion of 14 kb which removed genes coding for drug and metal resistances (7, 19). pKM101 has been extensively studied because it increases the susceptibility of cells to UV and chemical mutagenesis by coding for the *mucA* and *mucB* genes, which are analogs of the chromosomally encoded *umuD* and *umuC* genes (11, 26), and it has played a major role in the success of the Ames *Salmonella* strains used for detecting mutagens (22). The results reported here indicate that the conjugal transfer system of the clinically important IncN plasmids differs in many ways from that of the more widely studied IncF plasmids.

MATERIALS AND METHODS

Bacterial strains. Strains AB1157, TK610, and ES689 are described by Shanabruch and Walker (27). JC2926 is a *recA13* derivative of AB1157 obtained from A. J. Clark. JC7623 is a *recB21 recC22 sbcB15* derivative of AB1157 obtained from A. Clark. NG624 is a *trp lac rpsL31* strain obtained from E. Weinstock. GW4203 is a *srl-300*::Tn*10* derivative of AB1157 constructed in this study. Phages PRD1 and IKe were obtained from V. N. Iyer. λ *c*I857 *b*221 *rex*::Tn*5* (Km^r) *O*am29 *P*am80 (henceforth denoted λ::Tn*5*) was obtained from M. Fox.

Isolation of conjugation-deficient Tn*5* derivatives of pKM101. (i) Method A. A mid-log phase culture of ES689(pKM101) was infected with λ::Tn*5* (multiplicity of infection = 1.0) and incubated at 42°C for 40 min. An eightfold excess of log-phase AB1157 λ^r was added as a conjugal recipient. Cells were incubated on membrane filters (Millipore Corp.) overnight at 37°C, suspended, and plated on media selective for Km^r transconjugants. Colonies were screened for transfer deficiency by stabbing them onto lawns of NG624 on rich media. After 5 h of incubation at 37°C, plates were replicated onto selective media.

(ii) Method B. ES689(pKM101) was infected with λ::Tn*5* as above. Aliquots of cells were used to inoculate 30 ml of LB cultures containing 20 μg of kanamycin per ml and allowed to grow to stationary phase. Cells were lysed and purified plasmid DNA was used to transform strain TK610 to

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Km^r. Transformants were then screened for conjugation deficiency as described above. This approach was later modified such that infected cells were plated on solid media (24). Cells were spread in sufficient quantity to give rise to between 10^5 and 10^6 colonies per plate. This method was also used routinely to mutagenize nonconjugal plasmids.

(iii) Method C. A plasmid containing cloned fragments of pKM101 DNA was mutagenized with Tn*5*, using method B. Approximately 2 μg of the DNA of a plasmid containing Tn*5* was digested with a restriction endonuclease that does not cut within the transposon (*Eco*RI was generally used), and this DNA was used to transform the *recB recC sbcB* strain JC7623(pKM101) to Km^r. At least half of these Km^r transformants were determined to have incorporated the Tn*5* into the resident pKM101 by means of a double crossover (S. C. Winans and G. C. Walker, J. Bacteriol., in press).

Quantitative mating assays. Cultures of cells used as conjugal donors were grown to mid-log phase (approximately 5×10^8 cells per ml), and 30 μl of each was added to 3.0 ml of a mid-log-phase culture of NG624. Cells were collected by vacuum filtration onto Millipore filters, placed on prewarmed LB plates, and incubated at 37°C for 1 h, suspended in saline, and plated on selective media.

Complementation analysis of Tra^- pKM101 derivatives. (i) Transformation-mediated heterozygosis. Strains containing Tra^- pKM101 derivatives were rendered transformation competent by incubation for 30 min in 100 mM $CaCl_2$ at 0°C. We transformed into such strains various amounts (as indicated in Results) of the DNA of various Tra^- derivatives of pKM101. Cells were incubated on ice for 30 min, heat shocked at 42°C for 2 min, and incubated with vigorous aeration for 1 h in the presence of 1 ml of LB. A 0.1-ml amount of a 10-fold-concentrated culture of GW4203 was added as a conjugal recipient and cells were transferred to a 1.5-ml Eppendorf tube. Cultures were centrifuged for 5 min, and the cells were suspended and spotted onto 2.5-cm Millipore filters. These filters were placed on LB agar plates and incubated at 37°C. After 1 h the cells were suspended and plated on LB plates supplemented with 15 μg of tetracycline, 25 μg of ampicillin, and 25 μg of kanamycin per ml.

(ii) Conjugation-mediated heterozygosis. Each of the Tra^- pKM101 derivatives bearing a Tn*5* insertion in TRA I or TRA II was transformed into JC2926(pGW2137). A 10 μl portion of these strains was mixed pairwise on a sheet of Parafilm and deposited onto an LB plate. As soon as these drops had soaked into the plate, 10 μl of a mid-log-phase culture of JC2926(pGW2132) was added onto each spot. After 1 h of incubation, 20 μl of a mid-log-phase culture of strain GW4203 was added to each spot as a conjugal recipient of pGW2132. After a second incubation at 37°C lasting for 3 h, the spots were either streaked or replica plated onto LB plates supplemented with 25 μg of chloramphenicol and 15 μg of tetracycline per ml.

Quantitative complementation tests were performed in the same way except that cultures were spotted onto Millipore filters that had been placed on LB plates and prewarmed. After the final incubation period, filters were vortexed into saline and dilutions of this cell suspension were plated on selective media.

Construction of pGW1672. pGW1672 was made by using pKB444 (provided by K. Backman), which is a derivative of pBR322 containing, in place of the latter's *Pvu*II site, a "P_{lac}" promoter oriented such that transcription proceeds counterclockwise and, adjacent to it, a *Kpn*I site. pGW1672 was constructed by digesting pKM101 *traC1134*::Tn*5* with *Hin*dIII and *Kpn*I and cloning the region between the *Kpn*I-2 site (Fig. 1) and the proximal *Hin*dIII site of the Tn*5* into the gap in the vector created by digestion with the same two enzymes.

RESULTS

Minimal region of pKM101 DNA necessary for conjugal transfer. The portion of pKM101 DNA sufficient for conjugal transfer was determined by a comparison of the two conjugally proficient pKM101 deletion derivatives pGW1542 and pGW1514 (Fig. 1). The only DNA shared by them consists of 20.5 kb, including the *tra* region as defined by Langer et al. (18), and a smaller region containing functions required for replication (Rep). The smaller region is not required for conjugal transfer (7), so all sequences necessary for conjugal transfer must be confined to the 20.5-kb DNA fragment shared by pGW1542 and pGW1514.

Localization of three groups of *tra* genes by Tn*5* insertion mutagenesis. To localize genes within this 20.5-kb region of pKM101 DNA necessary for conjugal transfer, the plasmid was mutagenized with Tn*5* and screened for transfer-deficient derivatives. Tra^- insertions were isolated in three different ways as described above. A total of 111 Tra^- mutants were isolated, all of which were localized within a 15-kb region. Figure 1 shows a selected set of Tra^+ and Tra^- Tn*5* insertions that were mapped to this region along with one insertion of Mu d1(Ap *lac*) and one of Tn*9*. A quantitative analysis of the transfer deficiencies of the 28 Tra^- Tn*5* insertions shown in Fig. 1 is presented in Table 1. It is not yet known whether all of these insertions disrupt genes which are directly involved in conjugation or whether some insertions are merely polar on such genes. In the course of these studies, we have also isolated derivatives of the plasmid bearing insertions within this region that are Tra^+. These insertions define three groups of genes involved in transfer, denoted TRA I, TRA II, and TRA III. Some of the insertions outside these regions also affect conjugal efficiency to varying degrees (Table 1).

The left border of the TRA I region lies between Ω13::Tn*5* and Ω1114::Tn*5*, the right border of TRA I lies between Ω1134::Tn*5* and Ω1232::Tn*5*, and the left border of TRA II lies between Ω1232::Tn*5* and Ω1141::Tn*5*. The insertion Ω1232::Tn*5* inactivates a gene (*eex*) that is required for entry exclusion but not for conjugal transfer (34). The right border of the TRA II region lies between Ω1247::Mu d1(Ap *lac*) and Ω630::Tn*5*, the left border of TRA III lies between Ω1246::Tn*5* and Ω1143::Tn*5*, and the right border of TRA III lies between Ω1217::Tn*5* and Ω655::Tn*5*.

Of the 2.5-kb interval which separates TRA II from TRA III, 2.0 kb can be deleted without abolishing transfer. The construction of this deletion is illustrated in Fig. 2. Plasmid pGW1599 (Fig. 2) lacks the DNA between insertions Ω1247::Mu d1(Ap *lac*) and Ω1246::Tn*9*. pGW1599 transferred at the same 75-fold-decreased rate as did its parent pKM101Ω1246::Tn*9*. The other parent, pGW1514Ω1247::Mu d1(Ap *lac*), transferred with efficiency comparable to pKM101 itself, but only when the conjugal recipient expressed the Mu repressor to prevent zygotic induction of Mu d1(Ap *lac*). The reason for the 75-fold deficiency of pGW1599 and pKM101Ω1246::Tn*9* is not understood, but it is concluded that there are no genes within this 2.0-kb deletion which are absolutely required for transfer.

Genes necessary for sensitivity to donor-specific phage. pKM101 renders its host sensitive to bacteriophages IKe, PRD1, and PR4 (5, 16). IKe and PR4 have been found to bind to the tips of the plasmid-mediated sex pili (3). The

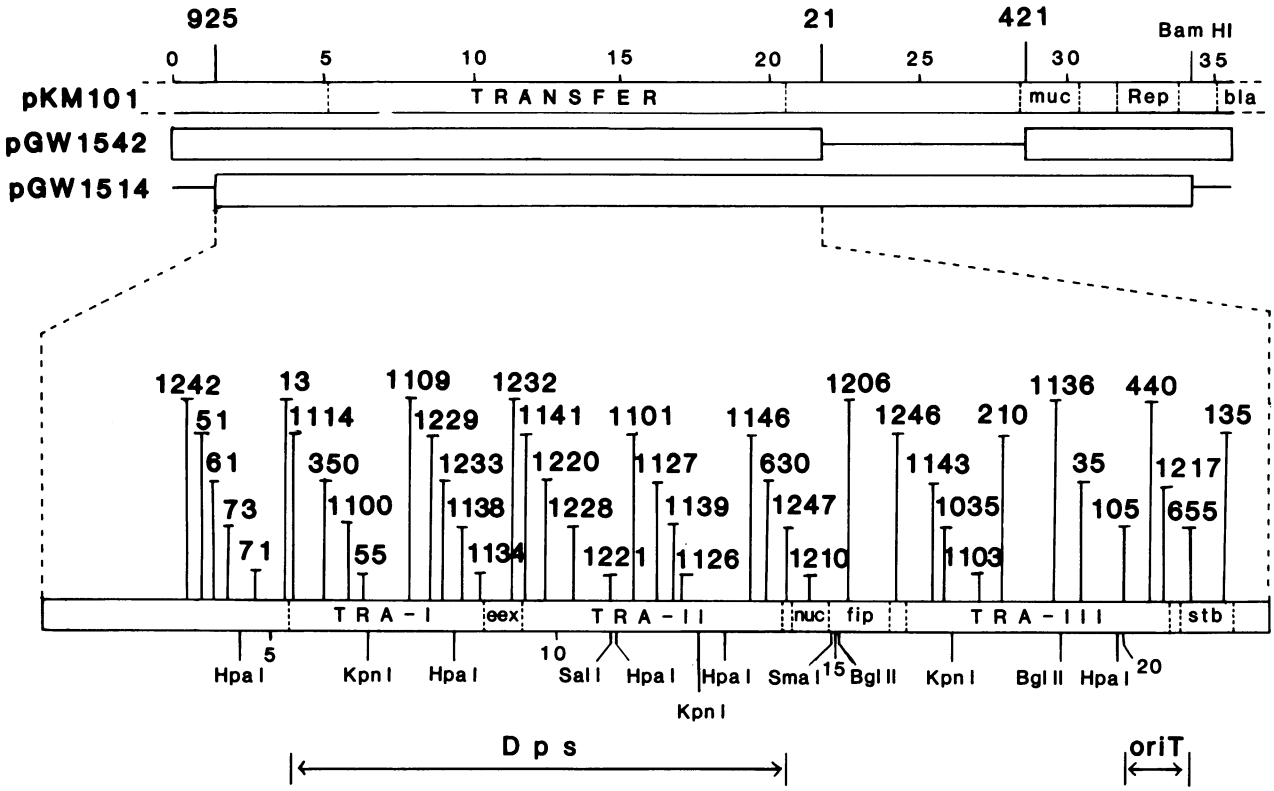

FIG. 1. Regions of pKM101 DNA necessary for conjugal transfer and sensitivity to donor-specific phage. Open bars denote pKM101 sequences present in deletion or cloned derivatives. Single horizontal lines indicate pKM101 sequences deleted. Numbers in boldface refer to Tn*5* insertions, except for 1247 and 1246, which refer to insertions of Mu dl(Ap *lac*) and Tn*9*, respectively. Dps refers to sequences required for sensitivity to donor-specific phage; *oriT* indicates the origin of conjugal transfer. pGW1542 was constructed by deleting the pKM101 DNA lying between insertions Ω21::Tn*5* and Ω421::Tn*5*. pGW1514 was constructed by deleting the smaller of the two *Bam*HI fragments of pKM101Ω925::Tn*5*.

genes sufficient for this phenotype, called Dps (for donor phage sensitivity), lie between Ω925::Tn*5* and Ω1247::Mu dl(Ap *lac*) (see Fig. 1). This is indicated by the fact that plasmids pGW1514 (Fig. 1), pGW1599 (Fig. 2), and pGW2137 (a clone of the *Bgl*II-A fragment, shown in Fig. 3), all mediate sensitivity to PRD1. All Tn*5* insertions within this region were tested for sensitivity to PRD1 (see Table 1) and it was found that all those insertions that were Tra^- were also Dps^r, whereas all Tra^+ derivatives were Dps^s. It is likely, therefore, that TRA I and TRA II consist largely, if not entirely, of genes necessary for pilus synthesis. A subset of these plasmids was tested for IKe sensitivity, and it was found that each of these plasmids displayed the same phenotype as it had when PRD1 was used.

Localization of the origin of transfer. In all systems of conjugation so characterized, transfer has been shown to be initiated from a fixed point, denoted *oriT* (29). Plasmids containing this site are efficiently mobilized if the host cell also contains the parental plasmid or a Tra^+ derivative. The *oriT* of pKM101 has been localized to a 1.2-kb region at or near the right end of TRA III. Derivatives of pACYC184 were constructed containing the *Bgl*II-A, *Bgl*II-E, or *Bgl*II-D fragment of pKM101, and each was tested for the presence of *oriT*. The plasmid containing *Bgl*II-D was efficiently mobilized, whereas plasmids containing *Bgl*II-A or *Bgl*II-E were not detectably mobilized. *oriT* must therefore lie within *Bgl*II-D. We isolated several Tn*5* insertion derivatives of this plasmid and have used them to create a series of deletion derivatives, two of which are illustrated in Fig. 3. pGW2204 was mobilized whereas pGW2202 was not. This indicates that *oriT* must lie to the left of Ω1284::Tn*5* and suggests strongly that it lies to the right of insertion Ω1281::Tn*5*. This conclusion is consistent with the finding (G. Coupland and N. S. Willetts, personal communication) that *oriT* is contained within the region flanked by the *Hpa*I-5 and *Hpa*I-6 sites shown in Fig. 4.

Complementation analysis of mutants in TRA III. *recA* strains harboring *tra* insertion derivatives of pKM101 were made transformation competent and transformed with plasmid DNA of other *tra* pKM101 derivatives. After allowing time for expression of the transformed plasmids' genes, GW4203 was added as a conjugal recipient. The cells were collected on Millipore filters, incubated at 37°C, suspended, and plated on selective media. Insertions in TRA III fall into four complementation groups: *traH*, *traI*, *traJ*, and *traK* (Table 2, Fig. 4). pKM101 *traI1136* and pKM101 *traK1217* were not tested for their ability to complement and were placed in different complementation groups because they are separated from each other by the three insertions in *traJ*.

Complementation of mutants in TRA I and TRA II. A second, more facile complementation test of mutants in TRA I and TRA II was developed which took advantage of two particular derivatives of pACYC184 containing pKM101 DNA: pGW2137, which carries the *Bgl*II-A fragment coding for pili, and pGW2132, which contains the *Bgl*II-D fragment carrying *oriT* (see Fig. 3). pGW2137 (which lacks *oriT* and therefore is not transferred itself) is used to complement the transfer deficiency of insertion mutations in TRA I or TRA II

TABLE 1. Characterization of insertion and deletion derivatives of pKM101

Plasmid[a]	Location (kb)[b]	Transfer efficiency (transconjugants/donor-h)	Dps[c]
pKM101		18.4	S
Insertion derivatives			
Ω1242	4.15	0.23	S
Ω51	4.05	5.6	S
Ω61	4.4	0.33	S
Ω73	4.45	0.11	S
Ω71	5.3	1.5	S
Ω13	5.3	1.9	S
traA1114	5.4	$<1.5 \times 10^{-5}$	R
traB350	5.9	$<1.5 \times 10^{-5}$	R
traB1100	6.45	$<1.5 \times 10^{-5}$	R
traB55	6.6	$<1.5 \times 10^{-5}$	R
traB1109	7.55	$<1.5 \times 10^{-5}$	R
traB1229	7.8	$<1.5 \times 10^{-5}$	R
traB1233	8.0	$<1.5 \times 10^{-5}$	R
traC1138	8.4	6.6×10^{-5}	R
traC1134	8.75	$<1.5 \times 10^{-5}$	R
eex1232	9.25	4.3	S
traD1141	9.4	$<1.5 \times 10^{-5}$	R
traD1220	9.7	$<1.5 \times 10^{-5}$	R
traE1228	10.3	$<1.5 \times 10^{-5}$	R
traE1221	11.0	$<1.5 \times 10^{-5}$	R
traE1101	11.35	$<1.5 \times 10^{-5}$	R
traE1127	11.7	$<1.5 \times 10^{-5}$	R
traF1139	12.0	$<1.5 \times 10^{-5}$	R
traF1126	12.2	$<1.5 \times 10^{-5}$	R
traG1146	13.5	$<1.5 \times 10^{-5}$	R
traG630	14.05	$<1.5 \times 10^{-5}$	R
Ω1247::Mu dl(Ap *lac*)[d]	14.15	6.5	S
nuc1210	14.5	28.5	S
fip1206	15.05	21.8	S
Ω1246::Tn*9*	15.9	0.3	S
traH1143	16.6	$<1.5 \times 10^{-5}$	S
traH1035	16.8	$<1.5 \times 10^{-5}$	S
traH1103	17.5	$<1.5 \times 10^{-5}$	S
traH210	17.9	$<1.5 \times 10^{-5}$	S
traI1136	18.7	1.5×10^{-5}	S
traJ35	19.4	$<1.5 \times 10^{-5}$	S
traJ105	20.0	$<1.5 \times 10^{-5}$	S
traJ440	20.5	$<1.5 \times 10^{-5}$	S
traK1217	20.6	2.4×10^{-4}	S
stb655	21.2	4.0	S
stb135	22.5	6.1	S
mucB1055	29.3	33.1	S
Deletion derivatives[e]			
pGW1542		1.1	S
pGW1514		37.8	S
pGW1599		0.43	S

[a] Unless otherwise stated, all insertions were made with Tn*5*.
[b] Position clockwise from *Eco*RI site of pKM101.
[c] Sensitivity (S) or resistance (R) to phage PRD1.
[d] Ω1247:Mu dl(Ap *lac*) is an insertion derivative of pGW1514 (Fig. 1). Matings with this plasmid utilized a recipient containing pGW600, a pBR322 derivative containing the Mu repressor.
[e] See Fig. 1 and 2.

and allow them to conjugate to a strain containing the *oriT* plasmid pGW2132 (Fig. 5); complementation of the two mutants being tested is assayed by testing for the mobilization of pGW2132 to a fourth strain.

Representative quantitative results are shown in Table 3. When JC2926(pKM101*traA1114*::Tn*5*)(pGW2137) or JC2926(pKM101*traD1141*::Tn*5*)(pGW2137), was added singly to JC2926(pGW2132), neither caused a high efficiency of mobilization (Table 3, lines 1 and 2), whereas when they were mated simultaneously into this strain the efficiency of mobilization was about 40-fold higher (line 3) and only 10-fold lower than that observed with a Tra$^+$ *mucB1055*::Tn*5* insertion (line 4). We believe that the background level of transfer of Cm^r observed when either of these donors was mated singly with JC2926(pGW2132) may be attributed to the low-frequency formation of a cointegrate between the mobilized plasmid and pGW2137, due to the presence of the insertion sequence IS*46* on each (A. M. C. Brown and N. S. Willetts, personal communication; Winans and Walker, unpublished data). Such cointegrates would be able to mobilize pGW2132, and because pGW2137 carries Cm^r, cointegrates would also be capable of transferring Cm^r directly to GW4203. In the qualitative tests summarized in Table 4, we monitored the growth of transconjugants on the selective plates at relatively short intervals after replica plating. These results were scored after about 8 h of growth, at which time we observed either confluent patches of transconjugants (scored as "++++") or a sparse field of 10 to 50 transconjugants (scored as "−"). In a few cases, as noted, intermediate numbers of transconjugants were obtained.

The results of the complementation analysis of the other TRA I and TRA II mutants are presented in Table 4.

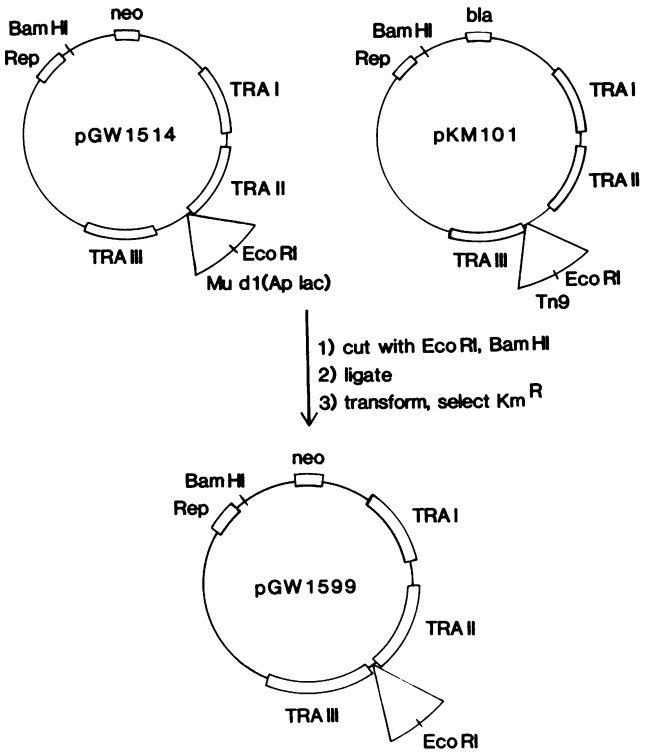

FIG. 2. Construction of a plasmid (pGW1599) containing a deletion of 2.0 kb of pKM101 DNA between TRA II and TRA III. This was done by fusing together fragments derived from two plasmids: pGW1514Ω1247::Mu dl(Ap *lac*) (pGW1514 is described in Fig. 1) and pKM101Ω1246::Tn*9*. Both plasmids were digested with *Eco*RI and *Bam*HI, ligated, and used to transform JC2926 to Km^r. The resulting chimeric plasmid contains two *Bam*HI-*Eco*RI fragments: the fragment of pKM101Ω1246::Tn*9* containing *neo*, TRA I, and TRA II, and the fragment of pGW1514Ω1247::Mu dl(Ap *lac*) containing TRA III and Rep. The point of insertion of the Tn*9* was 2 kb clockwise from the point of insertion of the Mu dl(Ap *lac*), and pGW1599 has a deletion of this interval.

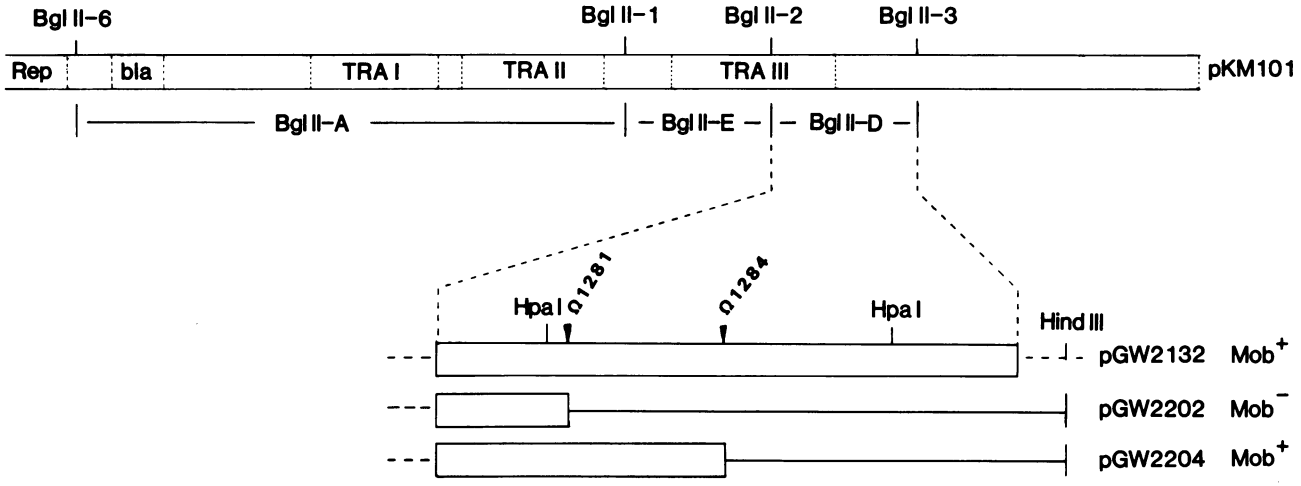

FIG. 3. Localization of *oriT*. The *Bgl*II-A, *Bgl*II-E, and *Bgl*II-D fragments of pKM101 were cloned into the *Bam*HI site of pACYC184, creating plasmids pGW2137, pGW2134, and pGW2132, respectively. Two Tn*5* insertion derivatives of pGW2132 (Ω1281::Tn*5* and Ω1284::Tn*5*) were used to construct deletion derivatives pGW2202 and pGW2204 by removal in vitro of the *Hin*dIII fragment of the corresponding insertion derivative. Open bars denote pKM101 sequences present in deletion or cloned derivatives. Single horizontal lines indicate pKM101 sequences deleted. Horizontal dashed lines indicate pACYC184-derived DNA.

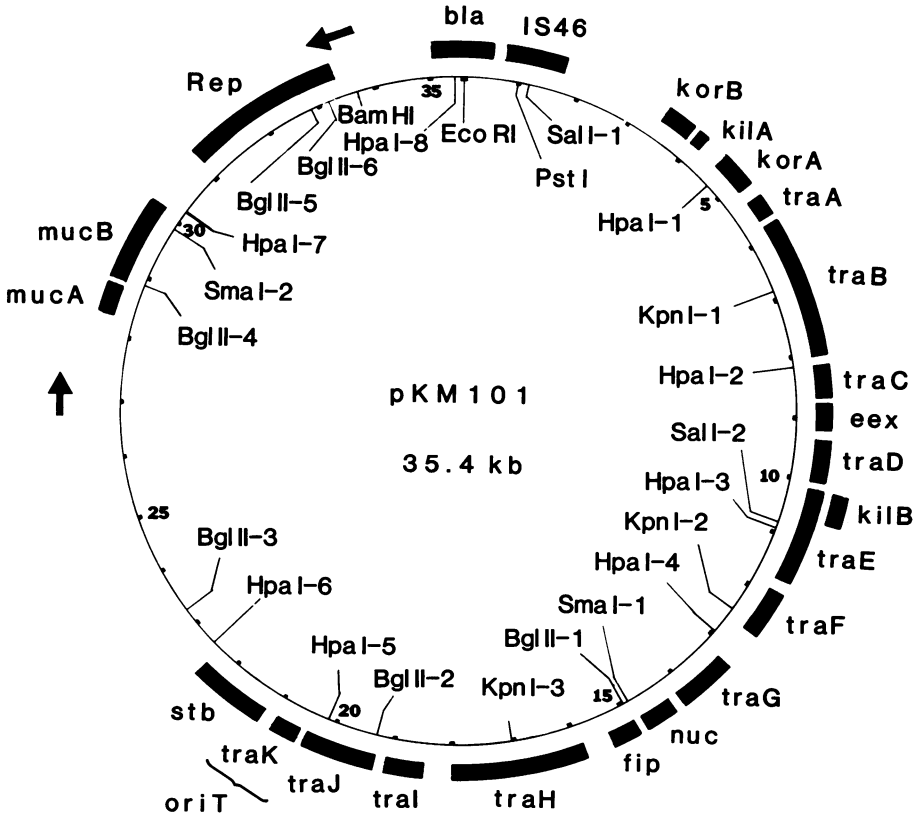

FIG. 4. Physical and genetic map of pKM101. Complementation groups *traA* through *traK* and *oriT* are described in the text. *bla* (β-lactamase), Rep (origin of replication), and inverted repeats (indicated by arrows) are described by Langer et al. (18). *mucA* and *mucB* (mutagenesis enhancement) are described by Perry and Walker (26). *nuc* (coding for a periplasmic endonuclease) is described by Winans and Walker (33). The following loci are described in the accompanying papers (34–36): *eex* (entry exclusion), *fip* (fertility inhibition of IncP plasmids), IS*46* (insertion sequence), *stb* (required for stability in recombination-proficient hosts), *kilA* and *kilB* (genes whose products are potentially lethal), and *korA* and *korB* (required to prevent lethality caused by *kilA* and *kilB*).

Insertions in TRA I (other than Ω1138::Tn*5* and Ω1134::Tn*5*) fall into two complementation groups: *traA* and *traB* (Fig. 4). Four additional complementation groups were found in TRA II: *traD*, *traE*, *traF*, and *traG* (Fig. 4). Since Tn*5* is usually strongly polar (17), one interpretation of the complementation groups described here and above is that they represent independent transcriptional units, each containing one or more genes.

Table 4 also shows, curiously, that both JC2926(pKM101Ω1138::Tn*5*)(pGW2137) and JC2926(pKM101Ω1134::Tn*5*)(pGW2137) are able to mobilize pGW2132 from JC2926(pGW2132) in the absence of any second strain to complement their transfer deficiencies and therefore were not amenable to complementation analysis by this method. We thus resorted to the complementation test involving transformation to analyze these insertions. Both of these plasmids complement either *traA1114*::Tn*5* or *traB1233*::Tn*5* (Table 5). We were unable to test whether Ω1134::Tn*5* and Ω1138::Tn*5* can complement each other, however. The complementation test requiring transformation can be used only if the resident plasmid is extremely deficient in conjugation. Although pKM101Ω1134::Tn*5* and pKM101Ω1138::Tn*5* are strongly conjugation deficient (Table 1), when we increased the sensitivity of the assay we found (Table 6) that both plasmids (unlike *traA* or *traB* mutants) retained a residual capacity to conjugate. This level was sufficiently high to preclude their use as recipients in the complementation test. Ω1138::Tn*5* and Ω1134::Tn*5* have provisionally been placed in a complementation group designated *traC*, although we have no indication that they are actually in the same complementation group. Probably the best way to resolve this ambiguity will be to identify the polypeptides coded by the genes in this region.

Suppression of *traC* lesions by neighboring Dps$^+$ cells. Strains containing both pKM101 *traC* mutants and pGW2137 were shown above (Table 4) to have an anomolous ability to mobilize pGW2132 from JC2926(pGW2132) into a conjugal recipient. We have found that strain JC2926(pKM101 *traC1138*::Tn*5*)(pGW2132) transferred pGW2132 at a very low level (about 10^{-6} per donor; data not shown), indicating, as expected, that mobilization of pGW2132 required the *traC* gene product. Since the *traC* gene product is synthesized only by the nonmobilizable plasmid pGW2137 in the original donor, it seemed most likely that it was somehow being

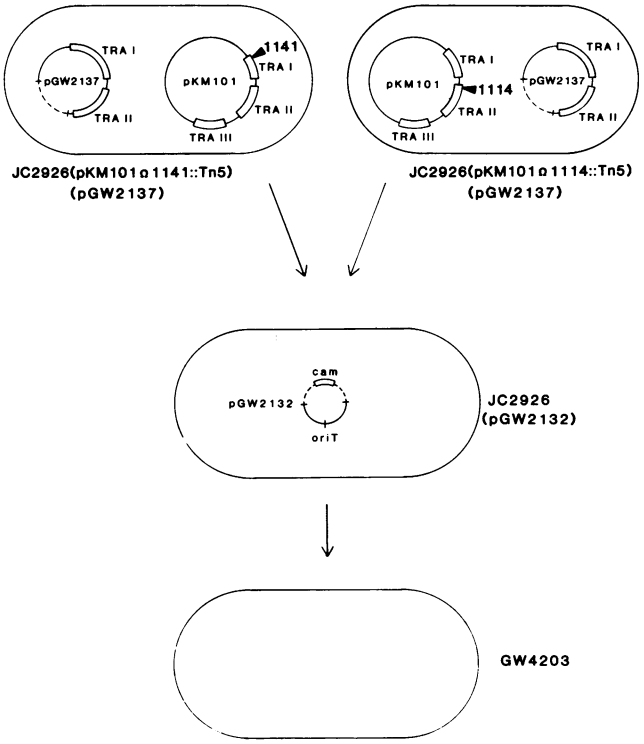

FIG. 5. Scheme used to delineate the complementation groups of Tn*5* insertions in TRA I and TRA II. In the example shown, the conjugation deficiencies of both pKM101Ω1114::Tn*5* and pKM101Ω1141::Tn*5* were suppressed by a coresident pGW2137, which is a derivative of pACYC184 carrying all of TRA I and TRA II. Two strains, each carrying one of these pKM101 derivatives and pGW2137, were mixed with JC2926(pGW2132) (Fig. 3) and deposited on an LB plate. The two pKM101 derivatives were transferred efficiently to JC2926(pGW2132), creating a transient heterozygote. (pGW2137 is not efficiently transferred, since it does not contain the pKM101 *oriT*.) If the insertions in the two pKM101 derivatives lie in separate complementation groups, then the pGW2132 in these recipients should be mobilized. After allowing this mating, GW4203 was added as a conjugal recipient, and mobilization of pGW2132 to GW4203 was monitored by selection for Cm^r Tc^r transconjugants.

TABLE 2. Efficiency of complementation of TRA III mutants during transformation-mediated transient heterozygosis

pKM101 derivative used for transformation	No. of Km^r Tc^r transconjugants per μg of plasmid DNA transformed[a]				
	traH1035[b]	*traH210*[b]	*traJ35*[b]	*traJ105*[b]	*traJ440*[b]
traH1143	1	17	ND	ND	5,400
traH1035	2	8	ND	ND	2,200
traH1103	2	11	ND	ND	380
traH210	5	16	540	1,300	600
traI1136	920	700	540	1,040	1,700
traJ35	1,300	470	12	13	3
traJ105	ND	ND	0	0	12
traJ440	ND	ND	10	6	2
traK1217	1,180	ND	4,100	3,400	16,500
traC1134	550	1,620	2,200	3,500	5,500
None	2	15	0	0	4

[a] Strains containing the given Tra$^-$ pKM101 derivatives were transformed with DNA of other Tra$^-$ pKM101 derivatives indicated in column 1. A total of 4×10^8 cells were used of strains containing Ω1035 or Ω210, whereas 8×10^8 cells were used of strains containing Ω35, Ω105, or Ω440. Strains containing Ω1035 and Ω210 were transformed with 3.0 μg of plasmid DNA and strains containing Ω35, Ω105, or Ω440 were transformed with 1.0 μg of plasmid DNA per ml. ND, Not determined.

[b] pKM101 derivative present in transformation recipient.

TABLE 3. Ability of two *tra*$^-$ derivatives of pKM101 having insertions in different complementation groups to mobilize pGW2132 during transient heterozygosis[a]

Donor[b]	Donors per filter	Cm^r Tc^r transconjugants per donor
traA1114	2.0×10^7	1.7×10^{-2}
traD1141	6.0×10^6	2.0×10^{-2}
traA1114 + *traD1141*	$1.0 \times 10^7 + 3.0 \times 10^6$	7.3×10^{-1}
mucB1055	5.0×10^6	7.2

[a] The donor strains indicated were spotted onto Millipore filters on an LB plate. Cultures of JC2926(pGW2132) and GW4203 were then added to filter. Mobilization of pGW2132 into GW4203 was monitored by selecting for Cm^r Tc^r transconjugants.

[b] All donor strains (JC2926) contain pGW2137 in addition to the pKM101 derivative specified.

TABLE 4. Efficiency of mobilization of pGW2132 by pairs of Tra$^-$ insertion derivatives of pKM101 during transient heterozygosis[a]

Tra insertion derivative	*traA-1114*	*traB-350*	*traB-1100*	*traB-55*	*traB-1109*	*traB-1229*	*traB-1233*	*traC-1138*	*traC-1134*	
TRA I										
traA1114	−									
traB350	+++	−								
traB1100	+++	−	−							
traB55	+++	−	−	−						
traB1109	+++	−	−	−	−					
traB1229	+++	−	−	−	−	−				
traB1233	+++	−	−	−	−	−	−			
traC1138	++++	++++	++++	++++	++++	++++	++++	++++		
traC1134	++++	++++	++++	++++	++++	++++	++++	++++	++++	
	traD-1141	*traD-1220*	*traE-1228*	*traE-1221*	*traE-1101*	*traE-1127*	*traF-1139*	*traF-1126*	*traG-1146*	*traG-630*
TRA II										
traD1141	−									
traD1220	−	+								
traE1228	+++	++++	−							
traE1221	++++	++++	−	−						
traE1101	++++	++++	−	−	−					
traE1127	++++	++++	+	−	−	−				
traF1139	++++	++++	+++	+++	++++	++++	−			
traF1126	++++	++++	+++	+++	++++	++++	−	−		
traG1146	++++	++++	+++	+++	++++	++++	++++	++++	−	
traG630	++++	++++	++++	++++	++++	++++	++++	++++	−	−

[a] Tetraparental matings were performed by using (i) strain containing plasmid indicated in top headings, (ii) strain containing plasmid indicated in column 1 (both of these strains also contained pGW2137), (iii) JC2926(pGW2132), and (iv) GW4203. Transfer of pGW2132 to GW4203 was monitored by selecting for Cm^r Tc^r transconjugants. "−", "++++," etc., indicate extent of transfer of Cm^r.

transferred to JC2926(pGW2132) in a form that was reusable. We have obtained other evidence that the *traC* gene product can indeed be transferred from one strain to another in a recyclable form. Specifically, the transfer deficiency of JC2926(pKM101 *traC*::Tn*5*) can be partially suppressed by exposing these cells, prior to mating, to a strain containing only plasmid pGW2137 (Fig. 3), which expresses *traC*, as well as the other genes in TRA I and TRA II, but which cannot be transferred itself. Strains carrying either pKM101 *traC1134*::Tn*5* or pKM101 *traC1138*::Tn*5* transferred Ap^r and Km^r to GW4203 at a rate between 550- and 1,620-fold higher in the presence of JC2926(pGW2137) than in its absence (Table 6). In a parallel experiment with fivefold fewer JC2926(pGW2137) cells, the level of suppression was about eightfold lower (data not shown). We have not determined which conditions are optimal for this suppression, so it is possible that more efficient suppression could be observed by varying experimental conditions. Other experiments demonstrated that none of the *tra* mutations described in this paper other than *traC* can be suppressed in this way by a Dps$^+$ strain.

To determine which genes on pGW2137 were necessary for suppression, strains containing pKM101 *traC*::Tn*5* derivatives were mixed with strains containing pKM101 derivatives having insertions in TRA I or TRA II in place of pGW2137. It was found that none of these strains could suppress a *traC* mutation, indicating that all of the genes of TRA I and TRA II must be functional. It is not known whether any genes present on pGW2137 other than these are also required. That all *tra* genes necessary for donor phage sensitivity are also needed for *traC* suppression suggests the possibility that the synthesis of functional sex pili by a second strain is necessary and sufficient for this unusual type of suppression of the transfer deficiency of *traC* mutants.

TABLE 5. Efficiency of complementation of a *traA* or *traB* mutant by pKM101Ω1134 and pKM101Ω1138 during transformation-mediated transient heterozygosis[a]

pKM101 derivative used as transformation donor	No. of Km^r Tc^r transconjugants obtained per μg of DNA transformed	
	traA1114[b]	*traB1233*[b]
pKM101Ω1138::Tn*5*	830	50
pKM101Ω1134::Tn*5*	330	40
traA1114	0	30
traB1233	980	0
None	0	0
traH1143	680	420

[a] A total of 4×10^8 transformation-competent cells of strains containing the *traA1114* or *traB1233* derivative of pKM101 were transformed with 3.0 μg of DNA of the Tra$^-$ pKM101 derivatives indicated in column 1. After heat shock and expression, GW4203 was added as a conjugal recipient and mating was allowed to occur on Millipore filters, followed by plating on selective media.

[b] pKM101 derivative present in transformation recipient.

TABLE 6. Suppression of the transfer deficiency of *traC* mutants of pKM101 by cells containing pGW2137[a]

Plasmid	JC2926(pGW2137) present in mating[b]	Km^r transconjugants/donor	Fold suppression[c]
traC1138	−	2.1×10^{-6}	NA[d]
traC1134	−	6.9×10^{-6}	NA
traC1138	+	3.4×10^{-3}	1,600
traC1134	+	3.8×10^{-3}	550

[a] Strains containing the plasmids indicated in column 1 were deposited on Millipore filters either alone or with JC2926(pGW2137). After 3 h these cells were vortexed, mixed with GW4302 (a Tc^r conjugal recipient), and deposited onto fresh filters. After a second incubation, they were suspended in saline and plated on media containing kanamycin and tetracycline.

[b] "+" indicates that 5.6×10^7 cells of JC2926(pGW2137) were added to the mating mixture.

[c] Transconjugants per donor in the presence of JC2926(pGW2137) divided by transconjugants per donor in the absence of JC2926(pGW2137).

[d] NA, Not applicable.

Preliminary efforts were made to determine whether the factor responsible for *traC* suppression could be isolated from JC2926(pGW2137). To date, we have been unsuccessful in efforts to suppress the transfer deficiency of *traC* mutants by using cell-free supernatants of JC2926(pGW2137) or preparations of isolated IncN sex pili (3). Nor did we observe suppression if Dps^+ cells were grown overnight on the underside of a Millipore filter and a strain containing a *traC* mutant plus a conjugal recipient were spotted on the upper side (the filter being placed on an LB plate). These preliminary results suggest that cell-to-cell contact is required for the suppression of *traC* lesions by a neighboring strain carrying pGW2137.

Relationship of *traE* to *kilB*. pKM101 *traE1228*::Tn*5* and pKM101 *traE1221*::Tn*5* (among others) were constructed by crossing the Tn*5* insertions from pGW1672, a recombinant plasmid carrying *traE*. pGW1672 contains *kilB*, a gene whose product is potentially lethal to the host and whose lethality is suppressed by two other plasmid-coded loci, *korA* and *korB* (Fig. 4) (35). A number of insertions were made in this plasmid and each was tested for *kilB* deficiency. The derivative pGW1672Ω1228::Tn*5* was determined to be *kilB*, whereas all other insertions tested, including pGW1672Ω1221::Tn*5*, were $kilB^+$. Ω1228::Tn*5* and Ω1221::Tn*5* do, however, lie in the same complementation group for conjugation, *traE*. The simplest interpretation of these results is that *traE* contains at least two cotranscribed genes, one of which is *kilB*. Since pGW1672 *traE1221*::Tn*5* is $kilB^+$, this insertion ought to be promoter distal from *kilB*. A second interpretation is that the *traE* complementation group contains one gene coding for a multifunctional protein. In this case, the amino-terminal fragment would have to be sufficient for the *kilB* phenotype.

DISCUSSION

The results described here indicate that the conjugal transfer region of broad-host range IncN plasmids, such as pKM101, differs in may ways from the conjugal transfer region of the more widely studied IncF plasmids, such as F. (i) The genetic organization and size of the *tra* regions of the two types of plasmids are very different. The *tra* genes of F are encoded in a single contiguous region of DNA approximately 35 kb long. In contrast, the *tra* genes of pKM101 are located in three discrete regions with a total length of between 16 and 20.5 kb. (ii) The origin of conjugal transfer (*oriT*) is found at one end of the *tra* region in each plasmid; however, in F, *oriT* is located at the end encoding the pilus genes, whereas in pKM101 it is located at the opposite end. (iii) The transcriptional organization of the two *tra* systems may be quite different. Of the 20 *tra* genes of F, 18 are transcribed in one operon (32). In contrast, the 28 *tra* Tn*5* insertion mutations that we characterized fell into 11 complementation groups. Since Tn*5*-induced mutations are usually strongly polar, these complementation groups may represent independent transcription units, although other models are possible. (iv) Although both plasmids code for genes mediating entry exclusion, the location of the genes is quite different. In F, the entry exclusion-mediating genes *traS* and *traT* lie within the long *tra* operon adjacent to the pilus genes and are cotranscribed with the *tra* genes that flank them. In contrast, in pKM101, the entry exclusion-mediating gene *eex* lies between two blocks of pilus genes. Tn*5* insertions in *tra* genes are not polar on *eex*, and an insertion in *eex* is Tra^+ (34), suggesting that *eex* is transcribed from its own promoter. (v) Transcription of the F *tra* operon is controlled by a positive effector, the product of the *traJ* gene; *traJ* mutants are pleiotropic, being deficient in both transfer and entry exclusion. To date, no candidate for an analog of *traJ* has been identified in pKM101. (vi) The sex pili of F have a thick flexible morphology, whereas the sex pili of IncN plasmids have a thin rigid morphology (4). (vii) The transfer efficiency of F is about the same whether mating is carried out on solid or in liquid media, whereas the efficiency of IncN plasmids is up to 10,000-fold higher on the former than in the latter (6).

In certain of these respects, the conjugal transfer system of pKM101 seems more closely related to that of the IncP plasmid RP4. Both plasmids have pili that have been designated thin and rigid (4). Both plasmids conjugate far more efficiently on solid media than in liquid (6). Furthermore, some (although not all) of the donor-specific phage that attack cells containing IncN plasmids also attack cells containing IncP plasmids (3).

Certain other similarities became apparent during the course of this work. First, although the size of the transfer region of RP4 is rather poorly defined, it is not unlikely that both plasmids have transfer regions of approximately equal size. The transfer genes of RP4 are localized in three regions (1), although no insertions have been isolated between two of these regions, so they may be contiguous. Likewise, the transfer region of pKM101 has at least three groups of transfer genes. Second, tra^- insertions in either plasmid are not polar upon extensive stretches of their respective transfer regions. This indicates that the transfer cistrons of RP4, like those of pKM101, may be transcribed from numerous promoters.

Despite these similarities, several major differences exist between IncN and IncP plasmids. First, transfer-deficient derivatives of pKM101 are not complemented by RP4 (data not shown). Second, whereas the three blocks of transfer genes of pKM101 are clustered together, those of RP4 are more widely dispersed around the plasmid. The two gaps that separate the three blocks of RP4 genes are 4 and 13 kb in length (versus 0.7 and 3.0 kb). Third, all entry exclusion-deficient insertions of RP4 are also tra^-, whereas no insertion in pKM101 having such a phenotype has been isolated.

To our knowledge, no observations have been reported of suppression of any transfer lesion by pilus-elaborating cells, analogous to the suppression of *traC* mutations described above. Studies concerning the physical properties of isolated F pili, however, may be of some relevance. Folkhard et al. (12) found that isolated sex pili which had been disaggregated into their subunits could be caused to reaggregate into either of two forms, depending on the conditions used. With a physiological buffer, pilin aggregated into a helical structure that was essentially identical to native pili, whereas in the presence of ether or low pH, pilin aggregated in a planar conformation which formed vesicles. The protein coats of various filamentous phage also seem to share this property (21). Folkhard et al. speculated that F pilin may exist in vivo in an equilibrium between a filamentous polymeric form and a monomeric form that is dissolved in the cell membrane. They further speculate that, after the pili contact female cells or male-specific phage, this equilibrium may shift in favor of the monomeric form, causing the pilus to shorten (see reference 8). Clark and Warren (9), in reviewing this study, entertained the possibility that F pilin might also dissolve, at least in part, into the membrane of the recipient. If this were true, then it seems possible that these pilin subunits might be available for reuse by the female. It is possible that the partial suppression of *traC* mutants of pKM101 by pili-elaborating cells might reflect such a phenomenon.

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