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Conjugal Transfer System of Plasmid RP4: Analysis by Transposon 7 Insertion

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We have begun an analysis in *Escherichia coli* of the conjugal transfer functions of the broad-host-range plasmid RP4. We have isolated 19 *tra* mutants of RP4, generated by insertion of transposon 7, and mapped their insertion sites by restriction endonuclease analysis. These sites fall into two separate regions on either side of the kanamycin resistance determinant. The transfer rates of the mutants range from 10% of that of RP4 to an undetectable level. Spot tests with the P-1 pilus-specific phages PRR1, Pf3, and PR4 and electron microscopic examination for pili have classified the mutants into several phenotypes consistent with their having normal, retracted, or no pili. Analysis of transient plasmid heterozygotes, created by P1 transduction, divided the *tra* mutants into a minimum of five complementation groups. Some of these groups contain more than one phenotypic class and may represent more than one gene because of the possible polar and deletion effects of Tn7 insertion.

We have recently mapped several genes on plasmid RP4 by using the ability of transposon 7 to insert itself at multiple sites on this plasmid (8). Transposon 7 (Tn7) is an 8.5×10^6-dalton DNA sequence, conferring trimethoprim (Tp) and streptomycin (Sm) resistances and capable of *recA*$^+$-independent transposition from one replicon to another. Its isolation and properties have been previously reported (6). (Tn7 was originally called TnC; see Cohen [14]).

We have mapped Tn7 insertions into RP4 by measuring the sizes of the restriction fragments given by the cleavage of such plasmids with restriction enzymes that have a single available site on RP4 itself, and at least one on Tn7. Insertions giving a particular phenotypic change were found clustered together on the circular map of RP4 at the site, we concluded, of the relevant gene.

Several Tn7 insertion mutants were found to have lost the conjugal transferability of RP4 (8). These mutants, although they gave three different classes of phage sensitivity phenotypes, were found to be clustered into a single small region of RP4. This region, corresponding in size to only one or two genes, was considerably smaller than the *tra* region of plasmid F. We have, therefore, extended this work by mapping more *tra* mutants of RP4. We present evidence here that RP4, unlike F and R plasmids of IncF II, has at least two widely separated *tra* regions.

We have also isolated some mutants with a reduced, rather than undetectable, rate of transfer. These may involve genes that promote conjugation between strains of *Escherichia coli* but are not essential to it, or they may be a consequence of polar effects of Tn7 insertion.

Plasmids, like RP4, belonging to incompatibility group P (P-1 in *Pseudomonas aeruginosa*), confer on their hosts the capacity to produce P-1 pili (9). It is generally assumed that such plasmid-determined pili are involved in the process of conjugal transfer. Various bacteriophages have been shown to adsorb to P-1 pili. PRR1, an RNA phage (26), attaches to the sides of the pili (9). The lipid-containing phages PRD1 (25), PR3, and PR4 (28), which are specific for P-, N-, and W-group plasmids (13), probably attach to the tips (10, 12). The filamentous phage Pf3 (28), specific for IncP plasmids only, is also thought to adsorb to P-1 pilus tips, but this has not been demonstrated. To determine whether or not our *tra* RP4::Tn7 plasmids determined P-1 pili, bacterial strains carrying them were tested for conferred sensitivities to these phages and examined directly in the electron microscope for the presence of pili.

We have assigned the RP4::Tn7 *tra* mutants to a minimum of five complementation groups. P1 transduction was used to form temporary plasmid heterozygotes, which were then scored for their ability to transfer the kanamycin (Km) resistance marker of RP4 to a suitable recipient under conditions of blocked P1 transduction.

We discuss the relationship between complementation groups and *tra* genes.

MATERIALS AND METHODS

Bacterial strains and plasmids. The strains of *E. coli* K-12 and plasmids used in this work are listed in Table 1.

Media. Minimal medium used was M9 (27) with glucose (2 mg/ml), required amino acids (200 μM), and thymine (20 μM). For labeling bacterial DNA, this medium was supplemented with Casamino Acids (Oxoid) at 5 mg/ml, plus [*methyl*-^{3}H]thymine (6.7 μCi/ml) or [2-^{14}C]thymine (1.2 μCi/ml) (Radiochemical Centre, Amersham), and, for *thy*$^+$ strains, deoxyadenosine (1 mM). Nutrient broth was Oxoid no. 2. Media were solidified with 1.2% agar (Oxoid no. 3).

Isolation and testing of Tn*7* derivatives of RP4. Tn*7* insertion mutants of RP4 were isolated as described previously (8); i.e., W3110T-TnC(RP4) was conjugated with J53, and trimethoprim (10 μg/ml)-resistant transconjugants of J53 were selected. Isolated colonies were purified and tested for the unselected resistance marker of Tn*7*, Sm (5 μg/ml), and those of RP4, ampicillin (Ap) (100 μg/ml), tetracycline (Tc) (10 μg/ml), and Km (25 μg/ml), using antibiotic ditch plates. These purified colonies were screened for conjugal transferability by simple plate matings (7); they were streaked into strain C600 spread over half a plate containing medium selective for Tp^r, Tc^r, or Km^r transconjugants. Clones that appeared transfer deficient were retested by mixing broth cultures of donor and recipient (grown at 37°C without and with shaking, respectively) in a 1:10 ratio, allowing conjugation for 1 h at 37°C without aeration, and then plating onto suitable selective media. Plasmids RP4 and pRP3 (see below) were used as *tra*$^+$ controls in parallel matings.

Sensitivity to phages. Bacteriophage PRR1 was provided by R. Olsen, Pf3 and PR4 were provided by V. Stanisich, and GU5 was provided by J. P. Hernalsteens. High-titer phage stocks were prepared as previously described (13). Spot tests for bacteriophage lytic activity were performed with nutrient plates overlaid with the test strain suspended in soft agar (0.6%) and incubated at 37°C overnight. The observation of any plaques at all was scored as sensitivity.

Complementation analysis of *tra* mutants. The RP4::Tn*7* strains with no detectable transferability were used to make P1 lysates, as previously described (6). These lysates were checked for sterility and titrated for plaque-forming units. Cultures of *tra* strains were grown with aeration in broth with 10 mM $CaCl_2$ to about 5 × 10^8 cells per ml. They were centrifuged, and the cells were concentrated 10-fold in the same medium. Samples (0.1 ml) were mixed with 0.1 ml of the chosen P1 lysate (diluted in P1 buffer [30]) to give a multiplicity of infection of about 1. After 15 min at 37°C to allow adsorption, 0.1 ml of sodium citrate (30 mg/ml in broth) was added. To each suspension was added 0.1 ml of strain C600 (the conjugative recipient), which had been grown in broth with aeration to about 5 × 10^8 cells per ml, centrifuged, and resuspended in one-tenth volume of broth with 10 mg of citrate per ml. After about 2 h at 37°C, 0.3 ml of the mixture was plated on minimal medium containing Km (25 μg/ml), citrate (10 mg/ml), and the nutritional requirements of C600.

Colonies appearing after 2 days of incubation at 37°C were counted. Some of them were purified on MacConkey medium containing Km. Lac$^-$ clones were tested for Tp and Tc resistance with ditch plates and for their ability to transfer Km^r to J53 by using the plate mating technique.

Test for surface exclusion. Exponentially growing cultures of W3110T$^-$(R702) and strains of the RP4::Tn*7* plasmids in J53 were mated in broth for 1 h at 37°C. Dilutions were plated on minimal medium selective for J53 plus 1 mg of sulfonamide per ml. R702 transfer into J53 and J53(RP4-δ1) was scored as surface exclusion (Sex)-negative controls, and transfer into J53(RP4) and J53(pRP3) was scored as Sex$^+$ controls.

Isolation of plasmid DNA. Cultures (7.5 ml) of *E. coli* strains, grown through three to four generations in the presence of radioisotope-labeled thymine, were lysed with Sarkosyl and centrifuged to equilibrium with ethidium bromide and CsCl as previously described (7). Isolated supercoiled DNA was dialyzed overnight against a continuous flow of 5 liters of TNE buffer [50 mM tris(hydroxymethyl)aminomethane–50 mM NaCl–1 mM ethylenediaminetetraacetic acid (pH 7.5)]. Plasmid copy numbers were estimated from the ratio of supercoiled-to-linear chromosomal DNA radioactivity, using the measured molecular weight of the plasmid and taking the *E. coli* chromosome as 2,500 megadaltons (Mdal).

Restriction endonuclease cleavage of DNA. Plasmid DNA in TNE buffer was supplemented with 0.125 volume of a 0.1 M $MgCl_2$–1 M tris(hydroxymethyl)aminomethane buffer (pH 7.5) plus 0.01 volume of the desired restriction enzyme (Miles Laboratories Ltd.), and incubated at 37°C for 1 h.

Sucrose gradient sedimentation analysis. The molecular weights of plasmids and their restriction fragments were estimated by sedimentation, together with suitable reference DNAs, through freeze-thaw-generated, isokinetic (ca. 5 to 20%) sucrose gradients. The preparation of such gradients and calculation of molecular weights have been described previously (7, 8).

Transformation. *tra*$^-$ transposition derivatives of RP4, chosen for electron microscopy, were transferred

TABLE 1. *Bacterial strains and plasmids used*

Strain	Characters[a]	Reference
W3110T$^-$	*thy deoC*	4
W3110T-TnC	*thy deoC* chromosomally inserted Tn*7*	8
J53	*proA metF* (λ)	4
C600	*thr leu thi lacY*	4
CR34-3 *rif*^{r}	*thr leu thi lacY thy deoC rpoB pil*	11
Plasmid		
RP4	IncP Ap^r Tc^r Km^r	15
RP4-δ1	IncP Ap^r Tc^r Tra Sex	17
R702	IncP Tc^r Km^r Sm^r Su^r Hg^r	18

[a] Symbols for bacterial and plasmid characters are according to Bachmann et al. (5) and Novick et al. (22), respectively.

to a strain lacking common pili (CR34-3 *rif*^{r}) by transformation, using plasmid DNA prepared as above and cells made competent according to the method developed by Jacob and Hobbs as described in reference 7. The recipient was, however, grown to late-log phase in broth rather than in P medium. Transformants were selected on nutrient media containing Ap (100 μg/ml), Tc (10 μg/ml), Km (10 μg/ml), or Tp (100 μg/ml), purified on plates containing Km, and then tested on ditch plates for all the RP4::Tn7 markers. The *thy* and rifampin (*rif*) resistance markers of CR34-3 *rif*^{r} were also tested. (Reduced-transfer mutants were transferred to CR34-3 *rif*^{r} by plate matings and then tested as above.)

All of the RP4::Tn7 *tra* mutants thus transferred to CR34-3 *rif*^{r} were crossed with C600 to measure their transfer frequency and thus check that this character had not changed. The phage spot tests were also repeated with these strains.

Electron microscopy. Bacterial strains were inoculated from static overnight cultures into fresh broth and grown for 3 h with shaking and then for a further 2 h without shaking. Equal volumes of this culture and PRR1 (ca. 10^{10} plaque-forming units/ml) were mixed in petri dishes to give PRR1-adsorbed pili, and equal volumes of culture and broth were mixed to give untreated control samples. A droplet of each of the mixtures was placed on carbon-coated grids held in forceps (clamped). The forceps were placed in covered petri dishes containing wet filter paper and incubated at 37°C for 10 min. Grids were then washed twice in 0.1 M ammonium acetate and negatively stained in 0.02% sodium phosphotungstate solution. For pilus counts, two grids were prepared from each sample by two different people. Pili on 50 cells were then counted in the electron microscope on each grid by the two people. Cells were counted at random, special care being taken with unlabeled samples not to miss short pili. Numbers presented are the sum of both counts (i.e., 100 cells examined for each sample). Pili were only counted if (i) negative staining was good enough clearly to reveal the cell edge and (ii) the pili projected from the cell edge. Listed counts are therefore comparative only and do not represent the total pili per cell.

RESULTS

Isolation of reduced-transfer mutants of RP4. Tn7 insertion mutants of RP4 are easily isolated as Tp^r transconjugants from a donor carrying Tn7 in its chromosome (see Materials and Methods). We have previously shown that about 2% of such transconjugants had lost conjugal transferability (8). (We assume that their initial transfer was mediated by either a cohabiting *tra*$^+$ RP4 or by conjugation mechanisms synthesized before Tn7 insertion disrupted a *tra* gene.)

We isolated more Tp^r transconjugants from W3110T-TnC(RP4) × J53 conjugations and screened them in a second cross for clones that had a Tp^r transfer frequency that was reduced in comparison with the majority of clones.

Five clones were found with reduced transfer frequencies of all the tested markers. Transconjugants from such matings were found to have acquired all the unselected markers of RP4 (Ap^r Tc^r Km^r) and Tn7 (Tp^r Sm^r). Thus, the five clones appeared to have plasmids that were reduced-transfer-frequency Tn7 transposition derivatives of RP4. The plasmids in these strains (see below) were designated pRP76, 89, 90, 91, and 92. (We have previously designated Tn7 derivatives of RP4 as RP4-TnC followed by a clone number [8]. For brevity and in conformity with the suggestion of Novick et al. [22], we now propose to designate them as pRP followed by the same clone numbers used previously.)

Transfer from reduced-transfer RP4::Tn7 plasmids is not due to reversion. We considered the proposition that the transfer frequencies observed for the five clones isolated above were a reflection of the reversion frequencies of completely transfer-deficient RP4::Tn7 mutants. Several transconjugants from each of the five mutants were therefore purified and tested for their conjugation frequency to a secondary recipient strain. We found that these transconjugants retained the characteristic transfer frequency of their parent strains and could not therefore be attributed to reversion.

Transposition of Tn7 from chromosome to chromosome. In the above isolation, we found two clones that had a markedly reduced Tp^r transfer frequency, but they transferred Ap^r, Tc^r, or Km^r at the same frequency as RP4 itself (i.e., about 10^{-3}/donor per h). Transconjugants selected for one of the RP4 markers carried the other unselected RP4 markers but not Tp^r. We therefore conclude that these two clones carried Tn7 in their chromosomes together with a free RP4 plasmid. Thus, in a small minority of cases, Tn7 has been mobilized by a plasmid from its chromosomal site in W3110T-TnC to a chromosomal site in J53. We have noted this phenomenon before (6). We do not know if a plasmid carrying an inserted Tn7 is a necessary intermediary in this process or not.

Previously isolated but unrecognized reduced-transfer RP4::Tn7 mutants. Since, in our initial isolation and screening of Tn7 insertion mutants of RP4, we did not look for reduced-transfer mutants (8), it seemed possible that we had isolated but overlooked such mutants. We therefore measured the conjugal transfer frequency of several mutants, especially those mapping on either side of the *tra* region already established. It was found that pRP26 and 46 (i.e., RP4-TnC26 and 46) had transfer frequencies of about 10^{-5} and 10^{-4} transconjugants/donor per h, i.e., reductions of 100- and 10-fold, respectively, compared to the RP4 or

pRP3 controls. Data on these mutants are therefore included in this paper.

Molecular weights of the *tra* RP4::T7 plasmids. ^{3}H-labeled plasmid DNA was prepared from the newly isolated reduced-transfer RP4::Tn7 clones and from previously isolated but unmapped *tra* clones (8). Each preparation was cosedimented with ^{14}C-labeled DNA from pRP1 (used as a reference) through a sucrose gradient (data not shown). Each clone was found to contain a single plasmid with a molecular mass of between about 43 and 45 Mdal (Table 2). This mass, as we found previously, is consistent with each plasmid being RP4 (36 Mdal) plus a single copy of Tn7 (8.5 Mdal) (6). Molecular mass data on the six *tra* plasmids reported previously (8) are also included in Table 2 for completeness.

Restriction enzyme mapping of Tn7 insertion sites. We mapped the Tn7 insertion site in each of the *tra* plasmids, using the restriction enzymes *Eco*RI, *Hin*dIII, and *Bam*HI, according to our previous method (8). We established in that paper that each of these three enzymes has one cleavage site on RP4, and that Tn7 has a single *Eco*RI site and two sites each for *Hin*dIII and *Bam*HI. The relative positions of these cleavage sites were also established (Fig. 1). Thus, one can calculate the site and orientation of each Tn7 insertion into RP4 from the sizes of the restriction fragments given by cleavage of the RP4::Tn7 plasmid by using (separately) any two of these enzymes. These data are given in Table 2, the plasmids being listed in the sequence of their calculated Tn7 insertion sites. This sequence is the best that we could assign from careful assessment and comparison of the restriction data. (As a consequence, Tn7 insertion sites in pRP28 [Fig. 1], 67, 69, and 77 [Fig. 1 and Table 2], are slightly different [0.1 Mdal] from those previously published [8].) The method does not allow us, however, to establish this sequence unequivocally.

The orientation of Tn7 in each of these plasmids was found to be the same as in all the (49) RP4::Tn7 plasmids we have analyzed to date. The orientation is such that the end of Tn7 closer to its *Eco*RI site (marked with an arrowhead) points clockwise on the map of RP4 as drawn in Fig. 1.

It can be seen from Fig. 1 that the *tra* muta-

TABLE 2. *Molecular masses of the intact and restriction enzyme-cloven RP4::Tn7 tra plasmids*

pRP no.	Molecular mass (Mdal)[a]	*Eco*RI fragments		*Hin*dIII fragments			*Bam*HI fragments			Tn7 insertion site[b]
		1	2	1	2	3	1	2	3	
46	44.0	36.7	7.8	31.9	10.5	2.1				30.9
92	44.3	36.0	8.3	32.7	9.8	2.0				30.2
76	45.3	35.3	9.2	33.1	9.5	1.9				29.5
90	44.8	35.1	9.4	33.0	9.6	1.9				29.3
89	44.0	34.9	9.6	33.3	9.3	1.9				29.1
67	43.6	34.0	10.5	34.7	7.9	1.9				28.2
69	42.7	34.0	10.5	34.1	8.3	2.1				28.2
68	44.2	33.9	10.6	35.0	7.6	1.9				28.1
74	44.9	33.8	10.7	35.1	7.5	1.9				28.0
66	44.6	33.4	11.1	35.1	7.5	1.9				27.6
81	44.1	33.4	11.1	34.7	7.7	2.0				27.6
75	43.5	33.3	11.2				25.5	18.1	0.8	27.5
77	43.7	33.3	11.2	35.1	7.4	1.9	25.3	18.4	0.8	27.5
79	44.0	33.2	11.3	35.6	6.9	2.0				27.4
78	44.7	33.1	11.4	35.7	7.0	1.9				27.3
26	43.7	33.0	11.5	35.8	6.8	1.9				27.2
91	44.5	23.0	21.5				28.6	15.0	0.9	17.2
80	43.7	22.5	22.0				29.2	14.6	0.7	16.7
82	43.8	22.5	22.0	31.7	10.8	2.0	29.2	14.6	0.7	16.7

[a] The molecular masses of the intact plasmids were measured by cosedimentation through sucrose gradients of each ^{3}H-labeled plasmid DNA with ^{14}C-labeled pRP1 DNA, taken as the reference molecule, with a mass of 45.0 Mdal (8). The restriction fragments of each RP4::Tn7 plasmid were similarly measured but ^{14}C-labeled RP4 DNA was used as the reference molecule. The RP4::Tn7 and RP4 DNA samples were mixed before treatment with restriction enzyme. RP4 is cut only once by each of the three enzymes used to give a linear 36.0-Mdal marker each time (8).

[b] The Tn7 insertion sites have been calculated from the restriction data as previously described (8), i.e., using two sets of restriction data to decide the orientation of Tn7 and its site in each plasmid. The number in the last column was generally calculated from the *Eco*RI fragment sizes and the position of the *Eco*RI site on Tn7 as 5.8 Mdal from its right-hand end (Fig. 1 [8]). For pRP91, 80, and 82, the sizes of the *Eco*RI fragments could only be approximately estimated since the two fragments merged into a single peak in the sucrose gradients. We therefore used the *Bam*HI data to calculate their Tn7 insertion sites (using the *Bam*HI sites on Tn7 given in Fig. 1). We obtained a standard deviation of 0.2 Mdal for repeated mapping estimates (8).

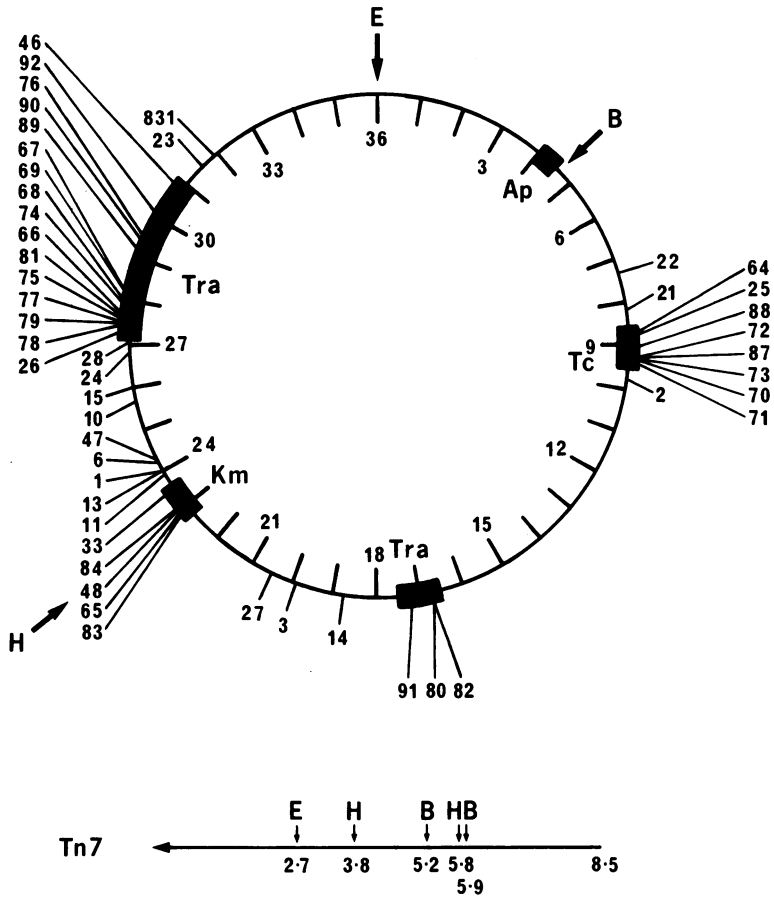

FIG. 1. *Map of Tn7 insertion and restriction enzyme susceptible sites on RP4 together with a diagram of Tn7 showing the approximate locations of its restriction enzyme sites (E = EcoRI, H = HindIII, and B = BamHI). The inner scale of the RP4 circle and the lower scale of the Tn7 map are marked in megadaltons. The Tn7 insertion sites on RP4 are marked with (pRP) clone numbers around the outside of the circle. Insertions that cause phenotypic changes to RP4 are marked on appropriately labeled blocks; those on the Tra blocks are described in this paper. The other insertions and the location of the gene determining Ap^r have been previously published (8). The arrowhead in Tn7 marks the end that we have found always points clockwise in Tn7 insertions of RP4.*

tions map at two quite separate regions, the one we have previously described and a new one at about 17 Mdal.

Plasmid pRP75. We reported previously (8) that pRP75 had a very low plasmid copy number (0.08 per chromosome equivalent compared to an average of 2.0 for the other RP4::Tn7 plasmids), although it was not detectably unstable. The previous mapping data, although unsatisfactory because of the low recovery of plasmid DNA, indicated that its Tn7 was inserted in the 27- to 28-Mdal region. During investigation of the cause of this low copy number, we transduced the plasmid (albeit at low frequency) from this strain (using phage P1 and Km^r selection) to another host and from there back into J53. The plasmid copy number in these strains was within the normal range. The data in this paper were obtained using these transduced strains. The previously approximate Tn7 map site was confirmed, but whereas the original strain conferred no phage sensitivities, the transductants conferred sensitivity to PR4 in spot tests (see below). Data on the cause of the low plasmid copy number in the original strain will be published separately.

Phage sensitivities. We tested all the *tra* mutants for their sensitivity to the phages PRR1, Pf3, PR4, and GU5 with simple spot tests. The results are given in Table 3. As the responses to PR4 and GU5 were always identical, we have not included a separate column for the latter. GU5 is morphologically indistinguishable from PR4 in electron micrographs and is

therefore probably closely related or identical to PR4. They both have a 65-nm-diameter head, which is probably icosahedral, and a 60-nm tail. It can be seen that the plasmids fall into several classes according to the pattern of phage sensitivities conferred.

P-1 pili determined by the RP4::Tn7 plasmids. We were interested to know how the phage sensitivities related to the number of pili determined by the *tra* RP4::Tn7 plasmids. Each plasmid was transferred to a strain lacking common pili by transformation or conjugation, as appropriate, and then retested for its resistance markers and characteristic undetectable or reduced-transfer frequency. The pili on 100 cells from each culture were counted in the electron microscope.

From the results given in Table 3 it is clear, firstly, that PRR1 treatment considerably enhanced piliation. Secondly, one can see that there is a general consistency between the results of the phage spot tests and the level of piliation. Thus, of the strains tested that were resistant to all the phages, most had no detectable pili (but 2 of 100 cells of pRP82 had one pilus each after PRR1 labeling). Those with PRR1 resistance but sensitivity to PR4 had detectable pili only after PRR1 adsorbance treatment. Fully phage-sensitive strains had pili under normal growth conditions as well. (It should be remembered that phage resistance indicated by spot test means only that the phage cannot form plaques and does not necessarily imply the complete lack of suitable attachment appendages.)

Complementation analysis. We used P1 transduction to form temporary heterozygotes of the *tra* RP4::Tn7 plasmids and then, in the presence of citrate (10 μg/ml) to prevent further transduction, tested them for conjugal transferability. Two control experiments were necessary for this technique. Firstly, we checked the effect of citrate on conjugation. In crosses using a *tra*$^+$ RP4::Tn7 plasmid, we found that citrate did not significantly alter the number of transconjugants (but did reduce the size of the colonies marginally). Secondly, we checked the inhibitory effect of citrate on transduction. We carried out a "complementation" experiment as described in Materials and Methods, but used P1 grown on a plasmid-free strain (P1.R$^-$). A few colonies were found. As these cannot be due to complementation, they must be due to P1 transduction of the *tra* plasmid to the final recipient (C600). Thus citrate does not completely abolish transduction under the conditions we have used. We

TABLE 3. *Conjugal transfer frequencies, phage sensitivities, and P-1 pili determined by the RP4::Tn7 tra plasmids*[a]

pRP no.	Transfer frequency	Phage sensitivity			Untreated pili		PRR1-adsorbed pili	
		PRR1	Pf3	PR4	Pili/cell	Piliated cells (%)	Pili/cell	Piliated cells (%)
46	10^{-4}	S	S	S	—	—	—	—
92	10^{-4}	S	S	S	0.01	1	1.56	65
76	10^{-6}	S	S	S	—	—	—	—
90	10^{-6}	S	S	S	0.05	4	0.74	49
89	10^{-5}	S	S	S	—	—	—	—
67	$<10^{-9}$	S	S	S	0.06	5	0.62	38
69	$<10^{-9}$	R	R	S	0	0	0.01	1
68	$<10^{-9}$	R	R	R	—	—	—	—
74	$<10^{-9}$	R	R	R	0	0	0	0
66	$<10^{-9}$	R	S	S	0	0	0.59	31
81	$<10^{-9}$	R	R	S	—	—	—	—
75	$<10^{-9}$	R	R	S	—	—	—	—
77	$<10^{-9}$	R	R	S	0.04	4	0.15	12
79	$<10^{-9}$	R	R	R	—	—	—	—
78	$<10^{-9}$	R	R	R	—	—	—	—
26	10^{-5}	S	S	S	—	—	—	—
91	10^{-5}	R	R	R	0	0	0	0
80	$<10^{-9}$	R	R	R	—	—	—	—
82	$<10^{-9}$	R	R	R	0	0	0.02	2
Control								
3	10^{-3}	S	S	S	0.14	14	0.83	53

[a] Transfer frequency is expressed as transconjugants per donor in nonaerated broth matings at 37°C for 1 h. Phage sensitivities were determined by spot tests. Pili, before and after treatment with PRR1, were estimated by electron microscopic examination of 100 cells. The plasmids are listed in their mapping order (Table 2). —, Not examined.

therefore performed a control experiment using the P1.R⁻ for each of the *tra* plasmids tested and subtracted the number of colonies found from each of the appropriate experiments. The results are shown in Table 4.

Crosses involving the reduced-transfer plasmid-containing strains could only be carried out by using them as P1 donors. They could not be used as P1 recipients (conjugal donors), because the frequency of transduction with a P1 plasmid stock was too low (about 10^{-5}) to expect a measurable increase in their inherent transferability by complementation. This expectation was confirmed. Reciprocal crosses involving these plasmids were, therefore, not possible, nor could we test for complementation between the reduced-transfer plasmids.

We tested about 100 transconjugants from these complementation tests (from crosses involving pRP78, 79, 80, and RP4-δ1) for their subsequent transferability. Only one was found to be Tra⁺ (and Tp^s). Thus, complementation, and only rarely recombination, are responsible for their appearance.

The results in Table 4 show that the plasmids we have isolated belong to a minimum of five complementation groups. Group 1 contains pRP82, 80, and 91; group 2, pRP26; group 3, pRP78 and 79; group 4, pRP77, 75, 81, 66, 74, 68, 69, and 67; and group 5, pRP89, 90, 76, 92, and 46. Because the reduced-transfer plasmids in group 5 could not be tested against one another and they cover about 2 MdaI (3 kilobases), we think that they probably constitute more than one complementation group. We consider below what these groups may mean in terms of *tra* genes.

Surface exclusion. We tested all the plasmids listed in Table 3 for their surface exclusion (Sex) effect on the entry of a second IncP plas-

TABLE 4. *Plasmid complementation data*[a]

pRP plasmid in P1 donor	pRP plasmid in P1 recipient (conjugal donor)												
	67	69	68	74	66	81	75	77	79	78	80	82	RP4δ1
46	*c.* 200									*c.* 900			
92	*c.* 200									*c.* 500			
76	*c.* 40									*c.* 80			
90	*c.* 1,000									*c.* 1,900			
89	*c.* 600									*c.* 1,200			
67	0	0	0	0	0	0	0	0	88				85
69	0	0		0				0					
68	0		0	0				0					57
74	0	0	0	0				0		234			
66	0				0			0	779	702		681	468
81	0					0		0					
75	0						0	0					
77	0	0	0	0	0	0	0	0		98			
79	51								0	0			
78				105				152	0	0		44	22
26	*c.* 900							*c.* 900		*c.* 900		*c.* 900	50
91										*c.* 500		0	
80											0	1	
82										175	0	1	1
RP4δ1	5									10		0	

[a] Crosses were performed as described in Materials and Methods. The plasmids are listed in mapping order (Table 2). The data shown are the number of Km^r colonies given per 10^9 P1 plaque-forming units after subtraction of the appropriate controls. When the P1 donor strain contained a reduced-transfer plasmid, these were: (i) transduction into J53(R⁻) to estimate the conjugal transferability of the resultant transductants (without complementation). This was always less than 1% of the yield from complementing crosses. (ii) Transduction with a P1.R⁻ stock to estimate the number of transductions of C600 due to the transductional recipient acting as a secondary source of transducing particles. This varied between 0 and 10% of the yield from complementing crosses (except for pRP80; see below). In crosses involving plasmids with no detectable transfer only control (ii) was found to be necessary, control (i), when tested, giving no detectable colonies. This is expected from the consideration that the number of secondary sources of transducing particles will be reduced by a factor (ca. 10^5) equal to the primary transduction frequency. When RP4-δ1 was the transductional recipient, its sensitivity to Km made control (ii) impossible (because Km was the selective drug throughout). When the transductional recipient contained pRP80, control (ii) gave consistently higher values than those of the other plasmids. We do not understand the reason for this effect, but it is not due to pRP80 having a higher transduction frequency than usual (about 5×10^{-6}). In contrast, RP4-δ1 has an unusually low transduction frequency (2×10^{-7}), which is responsible for the low numbers in the bottom line of this table. Numbers prefixed by *c.* were calculated from large colony numbers that could only be approximated.

mid, R702. The Sex$^+$ controls, RP4 and pRP3, reduced entry by a factor of about 20 to 50 compared to crosses with J53 or J53(RP4-δ1) (Sex$^-$ controls). None of RP4::Tn7 plasmids appeared to be Sex$^-$.

The deletion mutant RP4-δ1 has, however, lost its surface exclusion determinant (17). The data in Table 4 show that RP4-δ1 does not complement plasmids in group 1 (pRP91, 80, and 82) but does complement members of the other groups tested. Thus the 9.5-MdaI deletion (8) of RP4-δ1 (which is Km^s *tra* and *sex*) must cover approximately the 15- to 24-MdaI region of RP4. This revision of our previous conclusion about the site of the deletion (8), together with the consequent probable site of the *sex* gene, is considered below.

DISCUSSION

We have analyzed 19 *tra* mutants of RP4, including six previously reported (8), isolated as Tn7 insertion derivatives. The insertion sites in these mutants, mapped by restriction enzyme analysis, are located in two quite separate regions of RP4 (Fig. 1). RP4 therefore appears to have at least two *tra* regions rather than one as in plasmid F (2, 3, 23).

The function of the DNA between these two *tra* regions is of interest. We have generated plasmids in vitro, with specific deletions of this DNA (Barth, unpublished data). Together with the evidence on the site of the deletion in RP4-δ1, this shows that there are no genes essential to the viability of RP4 between the two *tra* regions. We therefore speculate that this region carrying the gene conferring Km^r may have been acquired (perhaps by transposition) by a primordial IncP plasmid in response to selective pressure, and that its insertion split a previously contiguous *tra* region.

The plasmid RK2, which is indistinguishable from RP4 in its properties (and may be identical to RP4; J. Beringer, personal communication), has also been the subject of in vitro deletions (16). These experiments, although they define only one *tra* region, are consistent with our map of RP4.

The reduced-transfer properties of some of the RP4::Tn7 plasmids described in this paper may be due to Tn7 insertion mutations of genes that promote, but are not essential to, conjugal transfer between *E. coli* K-12 strains. Olsen and Shipley (24) have shown that the transfer frequencies of RP1 in various inter- and intraspecific crosses vary by several orders of magnitude. Some of the *tra* gene products of promiscuous plasmids (like RP1 and RP4) may, therefore, be important in some crosses but not in others. The lack of any insertion mutants in the 28- to 29-MdaI region (Fig. 1) may thus be due not to genetic silence or lack of suitable Tn7 insertion sites there but to the choice of *E. coli* for the screening of *tra* mutants. The use of other genera may be necessary for a complete analysis of the *tra* regions of RP4.

Alternatively, the reduced-transfer phenotype may be due in some cases to Tn7 having a polar effect on distal genes within an operon into which it had been inserted. Such polar effects have been reported for Tn1 (19) and Tn10 (20). At present we cannot distinguish between the two mechanisms suggested for reduced-transfer mutants; both may be operating.

The unknown polar effects of Tn7 also limit our interpretation of the complementation groups in terms of *tra* genes. A second complicating factor is the fact that many RP4::Tn7 plasmids have apparently sustained a deletion of up to 2 MdaI (Table 2). The lack of reversion of such plasmids to Tra$^+$ (Grinter, unpublished observations) is consistent with our previous suggestion that Tn7 insertion sometimes causes a deletion in the recipient plasmid (8) and that this is at the site of the insertion. This point has been directly confirmed by heteroduplex analysis (G. Engler, M. Van Montagu, and J. Schell, personal communication). Thus, in each of our RP4::Tn7 plasmids, more than one *tra* gene may be mutant, so that we cannot simply deduce *tra* genes from complementation groups.

The mapping data suggest that complementation groups 2 (pRP26) and 3 (pRP78 and 79) may represent two adjacent *tra* genes (Fig. 1). Group 4 (pRP67, 69, 68, 74, 66, 81, 75, and 77) is complex in that it contains plasmids with various phage sensitivity phenotypes, indicated by spot tests (Table 3), although it covers a length of DNA consistent with being a single gene. Examination of pili determined by members of this group, by electron microscopy, also showed that there were major differences between them (Table 3). Thus pRP69 and 74 have apparently lost the ability to produce pili, but pRP67, 66, and 77 have retained this ability, although they produce significantly fewer pili than the *tra*$^+$ control when not labeled with PRR1. Interpretation of this complementation group must, therefore, await clarification of the complexities due to deletions and Tn7 polarity effects discussed above.

It should be noted that there were no inconsistencies between the mapping order of the mutants and their clustering into complementation groups.

The marked increase in the number of pili after PRR1 adsorption (Table 3) suggests that P-1 pili are retractile, being "locked" in an ex-

tended position by the adsorbed phages (12). The number of untreated PRR1-adsorbed pili seen by electron microscopy generally reflected the phage spot test sensitivities (Table 3). Visible PRR1 sensitivity apparently required that more than 31% of the cells be piliated after the PRR1 adsorption treatment.

Pf3 and PR4 are both thought to attach to the tips of P-1 pili (10, 12); therefore, we assume that retracted pili may still serve as attachment sites. The results with pRP82, 90, 67, 69, 66, and 77 are consistent with this, although we note that Pf3 and PR4 sensitivities do not always occur together. These phages therefore have different adsorption or infection specificities.

The level of piliation does not reflect the transfer frequency of each *tra* plasmid. For example, strains carrying pRP92 and 90 have about the same number of pili visible but they have a 100-fold difference in transfer frequency. Plasmid pRP67 gives full phage sensitivities and almost normal piliation levels but no detectable transfer (Table 3).

We concluded from the complementation data (Table 4) that the deletion in RP4-δ1 ($Km^s Tra^- Sex^-$) must cover approximately the 15- to 24-Mdal region of RP4. Stanisich and Bennett (29), from a study of deletion mutants, concluded that the Tra and Sex determinants of RP1 are closely linked (about 1 Mdal apart). As RP1 and RP4 are closely related, we predict that the Sex determinant must be at about 16 Mdal. We are now isolating Sex^- RP4::Tn*7* plasmids to test this prediction.

In conclusion, we have shown that at least five *tra* genes, grouped into two separate regions, are involved in the conjugal transfer system of RP4.

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