

## Transposon-Mediated Conjugational Transmission of Nonconjugative Plasmids

NANCY J. CRISONA,† JAN A. NOWAK, HARUKO NAGAISHI, AND ALVIN J. CLARK\*

*Department of Molecular Biology, University of California, Berkeley, California 94720*

When coresident with conjugative plasmid pNC21, the nonconjugative deletion F-prime pJC59, which retains the F transfer origin *oriT*, was transmitted to transconjugants at a frequency comparable to that of pNC21. In addition, pJC59 was transmitted as an independent plasmid, physically separate from pNC21, an example of plasmid donation. In contrast, two plasmids that are derived from F and deleted for the *oriT* site, pJC61 and pML31, were transmitted at frequencies  $10^4$  lower than that of pNC21. This low-frequency transmission was associated with the appearance of a new plasmid in the transconjugants. In the case of pML31, we determined that this new plasmid was a recombinant composed of pNC21 and pML31, the latter flanked by two copies of transposable element Tn3. We believe that this recombinant plasmid was formed as an intermediate in the transposition of Tn3 from pNC21 to pML31 and was the vehicle for conjugational transmission of pML31 genes by a process known as plasmid conduction.

Conjugational transmission of plasmids requires cell surface interactions, called specific contacts, between donor and recipient cells. In addition, it requires three DNA metabolic processes: mobilization in the donor, replication in the recipient, and transfer between the two cells. By preparing plasmid DNA for transfer, mobilization provides the transition between the vegetative processes of maintenance and the conjugational process of transfer. After transfer, plasmid DNA undergoes replication, consisting of recipient conjugal DNA synthesis and circularization, in preparation for the vegetative state.

Bacterial plasmids are classified as conjugative if they carry the genes required to establish specific contacts between donor and recipient cells. Such plasmids may also be transmissible by conjugation when they carry the genes required to effect the mobilization, transfer, and replication of their own DNA. These genes can be distinguished as *cis* or *trans* acting (8). The *cis*-acting genes, or more properly *cis*-acting sites, include, for example, *oriT* on F (11) and *bom* on ColE1 (40). These are thought to be sequences at which the products of the other genes act to promote transmission (8).

Nonconjugative plasmids may arise either as mutant derivatives (e.g., *FtraG* and pML31) of conjugative plasmids or as natural isolates (e.g., ColE1 and pSC101). Both classes can be tested for transmissibility when they and a suitable conjugative plasmid cohabit a cell. Many studies

on the transmission of nonconjugative plasmids in the presence of conjugative plasmids have been carried out (for a review, see reference 8). Kopecho and Cohen (27), for example, found that a *recA* mutant strain carrying conjugative plasmid pSC50 and tetracycline resistance-determining nonconjugative plasmid pSC101 transmits tetracycline resistance at a very low frequency relative to the transmission of the antibiotic resistances determined by the genes of pSC50. Furthermore, the rare Tc<sup>r</sup> transconjugants all inherit a mutant form of pSC101 carrying the transposable element Tn3 derived from conjugative plasmid pSC50. Crisona and Clark (11) discovered that *recA56* transconjugant progeny containing pSC101::Tn3 and pNC21, a conjugative plasmid similar if not identical to pSC50, can be high-frequency donors of tetracycline resistance. They hypothesized that the Tn3 insertion element carries a *cis*-acting site which increases the transmission frequency. Subsequent work with pSC101 and its derivatives (J. A. Nowak, N. J. Crisona, H. Nagaishi, and A. J. Clark, in preparation) and other plasmids (this report) has shown this not to be the case.

We have studied the transmission of three nonconjugative derivatives of transmissible conjugative plasmid F. Two of the plasmids are nonconjugative because they are deleted for all of the genes required for specific contact formation, which lie on restriction endonuclease *EcoRI* fragments 6, 15, 1, 17, 19, and 2 of F (1, 36). In one of these plasmids, pJC61, the segment of F between 52.7 kilobases (kb) and 16.3 kb on the standard map of F has been replaced by a

† Present address: Department of Zoology, University of California, Berkeley, CA 94720.

152-kb segment of the *Escherichia coli* chromosome carrying *argG* (21, 22). In the second plasmid, pML31, the very large segment of F between the *EcoRI* sites at 49.3 and 40.3 kb has been replaced by an *EcoRI* fragment carrying a gene derived from *Staphylococcus aureus* which determines kanamycin resistance (28). In the third plasmid, pJC59, only some of the genes involved in surface contact formation have been deleted, and a bacterial segment carrying *argG* has been substituted. In addition, pJC61 and pML31 are deleted for a required *cis*-acting site, *oriT*, located on *EcoRI* fragment 6 of F (42). Our objective was to determine whether transmission of these plasmids could be promoted by pNC21 and, if so, whether the presence of *oriT* affects the nature of this transmission. We found that transmission of pJC61 and pML31 genes was always associated with the presence of cointegrate plasmids in the transconjugants, indicating that transfer of these plasmids required recombination with the conjugative plasmid. The nature of the cointegrates and their component plasmids led us to suggest that the recombination required for the transmission of the non-mobilizable plasmids' genes occurred as a step in one possible pathway of transposition of transposon Tn3. Finally, we concluded that although a cointegrate plasmid was a mobilizable mutant form of the original nonconjugative plasmid, the insertion of Tn3 alone into the nonconjugative plasmid was not sufficient to make it mobilizable.

## MATERIALS AND METHODS

**Bacterial strains.** Except for strain constructions, all crosses employed both donors and recipients which carried *recA56*. To facilitate serial transmission of plasmids, five *recA56* strains which different nutritional properties were employed. These are listed in Table 1.

The experiments all used plasmid pNC21 as the conjugative plasmid for the mobilization of nonconjugative plasmids. pNC21 was ultimately derived from JC7127, which was sent to us by S. Cohen as an unidentified strain carrying R1-19. This strain is, however, not kanamycin resistant as it should be if it carried R1-19. This is consistent with the finding of Kopecko and Cohen (27): that what had been called R1-19 in several previous papers was actually a deletion mutant, pSC50, which had lost the *kan* gene(s). The *EcoRI* fragment pattern of plasmid DNA from JC7127 after agarose gel electrophoresis, however, showed some differences from the fragment pattern of pSC50 (27). We were able to detect, in crosses of JC7127, Cm<sup>r</sup> transconjugants which inherited plasmids with *EcoRI* fragment patterns identical to that of pSC50 (27). We derived a deletion mutant, pNC21, one such plasmid. We derived a reliable source of pNC21 by first crossing JC7127 with JC10117, a derivative of AB1157 carrying plasmid pRS5 (36). A Cm<sup>r</sup> [Tet<sup>r</sup>] transconjugant which carried both pNC21 and pRS5 (JC9463) was crossed with *recA56* strain JC7199. As all Cm<sup>r</sup> transconjugants proved to carry pRS5 in addition to pNC21, one (JC9469) was crossed with JC7607 (ethyl methane sulfonate-induced *recB*<sup>+</sup> back-mutant of JC4695 [37]). One Cm<sup>r</sup> [Arg<sup>+</sup> Met<sup>+</sup>] transconjugant (JC10896) was found to be Tet<sup>r</sup> and was crossed with *recA56* strain JC7199. One Cm<sup>r</sup> [Leu<sup>+</sup> Trp<sup>+</sup>] transconjugant (JC10897) was shown to have only one plasmid

TABLE 1. Genotype of five *recA56* strains

Strain	Gene symbol <sup>a</sup>																					
	<i>ara</i>	<i>arg</i>	<i>gal</i>	<i>his</i>	<i>ilu</i>	<i>lac</i>	<i>leu</i>	<i>mal</i>	<i>met</i>	<i>mtl</i>	<i>nal</i>	<i>pro</i>	<i>pyr</i>	<i>rec</i>	<i>rps</i>	<i>sup</i>	<i>thi</i>	<i>thr</i>	<i>ton</i>	<i>trp</i>	<i>tsx</i>	<i>xyl</i>
JC2924 <sup>b</sup>	14	E3	K2	4	+	Y1	6	A1	+	1	+	A2	+	A56	L31	E37	1	1	+	+	33	5
JC7133 <sup>c</sup>	+	G6	+	+	+	Y1 or Z4	6	A1	+	2	+	+	B <sup>d</sup>	A56	L104	+	+	+	A2	+	315	7
JC7199 <sup>e</sup>	+	G6	+	1	+	— <sup>d</sup>	+	A1	C58	+	+	+	+	A56	— <sup>d</sup>	+	+	+	+	+	5	+
JC7214 <sup>f</sup>	+	G6	+	+	+	Y1 or Z4	+	A1	+	2	A301	+	+	A56	E310, L104	+	+	+	A2	+	315	7
JC7532 <sup>g</sup>	9	+	K2	4	D145	Y1 or Z4	+	+	E46	1	+	A2	+	A56	L8 or L9	E44	1	+	1	3	3	+

<sup>a</sup> Gene symbols are those shown on the standard map of *E. coli* (3). Phenotype abbreviations employed for auxotrophic characters below are as follows: Arg, arginine; Thr, threonine; Leu, leucine; TL, threonine and leucine; Pro, proline; His, histidine; Met, methionine; IV, isoleucine and valine; Trp, tryptophan; Pyr, pyrimidine; —, unable to synthesize or utilize; +, able to synthesize or utilize.

<sup>b</sup> Reference 38.

<sup>c</sup> Reference 21.

<sup>d</sup> Allele number unknown.

<sup>e</sup> Constructed by M. Guyer as follows. AT2699 (14) was exposed to nalidixic acid, and a spontaneous resistant mutant JC7059 was selected. JC7059 was then crossed with *recA56* donor JC5088 (2), and Thy<sup>+</sup> [IV<sup>+</sup>] transconjugants were selected. One of these (JC7199) had also become nalidixic acid sensitive.

<sup>f</sup> JC7214 is an eight-step derivative of JC411 (chart 9 of reference 2) constructed by M. Guyer as follows: (i) JC7029, a spontaneous Su<sup>-</sup> mutant of JC411; (ii) JC7120, a Met<sup>+</sup> [Sm<sup>r</sup>] transconjugant from the cross of Hfr strain CYA289 (4) with JC7029, inheriting Ura<sup>+</sup> of CYA289; (iii) JC7125, a His<sup>+</sup> [Sm<sup>r</sup> IV<sup>+</sup>] transconjugant from the cross of JC5088 (Hfr) with JC7029, inheriting *recA56* of JC5088; (iv) JC7133, a spontaneous T6<sup>r</sup> mutant (*tsx-315*) of JC7125; (v) JC7202, a spontaneous Leu<sup>+</sup> revertant of JC7133; (vi) JC7207, a spontaneous Ura<sup>+</sup> revertant of JC7202; (vii) JC7210, a nitrosoguanidine-induced Spc<sup>r</sup> mutant (*rpsE310*) of JC7207; (viii) JC7214, a spontaneous NaI<sup>r</sup> mutant (*nalA301*) of JC7210.

<sup>g</sup> Constructed by L. Margossian as follows. AB2277 (13) was exposed to trimethoprim, and a spontaneous thymine-requiring mutant (JC7896) was selected. JC7896 was crossed with *recA56* donor JC5088 (2), and Thy<sup>+</sup> [Thr<sup>+</sup> Sm<sup>r</sup>] transconjugants were selected; a Rec<sup>-</sup> UV<sup>+</sup> transconjugant was called JC7532.

(pNC21) and is now our standard source of this plasmid.

The non-conjugative-plasmid-carrying strains together with their pNC21 derivatives are listed in Table 2. One of these, JC10898, was the donor from which several transconjugant progeny characterized in Results were derived. DNA from some of these transconjugants was used to transform the recipient, JC7199. A summary of the plasmids in these strains is in Table 3.

The following abbreviations for antibiotics are used in conformity with the recommendations of Novick et al. (33): Ap, ampicillin; Cm, chloramphenicol; Km, kanamycin; Sm, streptomycin; Tc, tetracycline. Superscripts "r" and "s" used with these abbreviations stand for "resistant" and "sensitive," respectively.

**Bacterial matings in liquid medium.** Donor and recipient cultures were grown in Luria broth with aeration (43), plus antibiotics if appropriate, to a concentration of  $1 \times 10^8$  to  $2 \times 10^8$  cells per ml (a reading of 50 with a 660-nm red filter in a Klett-Summerson colorimeter). Cells grown in the presence of antibiotics were harvested, washed once with L broth, and resuspended in L. Donors and recipients were mixed in a 1:10 ratio and mated for 1 h at 37°C without aeration. The matings were terminated by mixing the mating mixtures at high speed in a Vortex mixer for 1 min. This mating mix was subsequently centrifuged, and the cells were suspended in 56/2 buffer (41) before dilution and plating on selective media.

Antibiotics were used at the following concentrations: kanamycin (kanamycin sulfate, Sigma Chemical Co.), 25 µg/ml; chloramphenicol (Sigma), 25 µg/ml; ampicillin (Alpen-N sodium ampicillin, Lederle Laboratories), 50 µg/ml; tetracycline (45-µg/ml Achromycin, tetracycline hydrochloride, Lederle), 15 µg/ml.

**Acridine orange curing.** Standing, overnight cultures in Luria broth, plus antibiotics if appropriate, were diluted 10<sup>7</sup>-fold into 3-ml samples consisting of pH 7.8 L broth and pH 7.8 L broth containing 10 or 25 µg of acridine orange per ml. The tubes were covered with foil to exclude light and were incubated at 37°C overnight with shaking.

Cells from the L broth sample and from the highest-concentration acridine orange sample at which there was growth were streaked onto L plates. To test for the loss of plasmids carrying antibiotic resistance, single colonies were transferred onto L plates and

replicated onto L plates containing the appropriate antibiotic. For F' strains, colonies were patched onto minimal medium plates and tested for the inheritance of the phenotype determined by the F' plasmid.

**Preparation of plasmid DNA.** Plasmid DNA was prepared either by the method of Meyers et al. (32) as described or by a modification of the Clewell and Helinski (9) method obtained from V. Hershfield and described below.

Cultures were grown in L broth, plus antibiotics if appropriate, at 37°C with shaking to a Klett reading of 100 ( $\sim 5 \times 10^8$ ) cells per ml. The cells were harvested and washed once with TES (50 mM Tris [pH 8.0], 5 mM EDTA, 50 mM NaCl). Cells from a 250-ml culture were suspended in 3 ml of 25% sucrose in 50 mM Tris (pH 8.0) in preparation for lysis. A 0.6-ml portion of a 10-mg/ml solution of lysozyme in 0.25 M Tris (pH 8.0) was added, and the cells were incubated on ice for 10 min, after which 1.2 ml of 0.25 M EDTA (pH 8.0) was added and the cells were incubated on ice for another 10 min. The cells were lysed by the addition of 4.8 ml of lytic mix (0.0625 M EDTA, 0.05 M Tris [pH 8.0], and Triton X-100 in a final concentration of 1%). A cleared lysate was prepared by centrifugation at 39,100 × g and 4°C for 30 min in a Sorvall RC2-B centrifuge without braking. Each 3 ml of the cleared lysate was brought to 3.8 ml with TES, and 3.65 g of CsCl was

TABLE 3. Summary of plasmids present in transconjugants derived from JC10898 and transformants derived from the transconjugants

Strain	Plasmid		Cointegrate
	pML31::Tn3	Original conjugative	
JC11625 <sup>a</sup>	pNC12	pNC21	pNC14
JC11626 <sup>a</sup>	pNC13	pNC21	pNC15
JC11130 <sup>a</sup>	pJC628	pNC21	pJC630
JC11131 <sup>a</sup>	pJC629	pNC21	pJC631
JC11128 <sup>b</sup>	pNC12	pNC21	ND <sup>c</sup>
JC11129 <sup>b</sup>	pNC12	pNC21	pNC14

<sup>a</sup> Km<sup>r</sup> [Pro<sup>+</sup> TL<sup>+</sup>] transconjugant derived by crossing JC10898 with JC7199.

<sup>b</sup> Km<sup>r</sup> Ap<sup>r</sup> transformant of JC7199, using DNA isolated from JC11626.

<sup>c</sup> ND, Not detectable.

TABLE 2. Strains carrying nonconjugative plasmids alone or together with conjugative plasmid pNC21

Nonconjugative plasmid	Strain carrying nonconjugative plasmid alone	Reference	Strain carrying nonconjugative plasmid and pNC21	Genetic background	Reference
None			JC10897	JC7199	This paper <sup>a</sup>
pJC59	JC7247	21	JC11616	JC7133	This paper <sup>b</sup>
pJC61	JC7249	21	JC10862	JC7133	This paper <sup>c</sup>
pML31	JC10471	This paper <sup>d</sup>	JC10898	JC2924	This paper <sup>e</sup>

<sup>a</sup> See text.

<sup>b</sup> A Cm<sup>r</sup> [Arg<sup>+</sup> His<sup>+</sup> Met<sup>+</sup>] transconjugant from a cross of JC10897 and JC7247.

<sup>c</sup> A Cm<sup>r</sup> [Arg<sup>+</sup> His<sup>+</sup> Met<sup>+</sup>] transconjugant from a cross of JC9469 and JC7249.

<sup>d</sup> A Km<sup>r</sup> transformant of JC2924, using pML31 DNA purified from an unnamed pML31 derivative of CR34 received from D. Helinski.

<sup>e</sup> A Cm<sup>r</sup> [Km<sup>r</sup> Met<sup>+</sup>] transconjugant from a cross of JC10897 and JC10471.

added for each 3.8 ml. Ethidium bromide was then added to a final concentration of 500  $\mu\text{g/ml}$  by using a 10-mg/ml solution of ethidium bromide in TES (pH 7.5). The final density of the samples was 1.55  $\text{g/cm}^3$ . The gradients were centrifuged in a Beckman type 50 rotor for at least 30 h at 36,000 rpm and 15°C without braking. Plasmid DNA was collected with a syringe and 20-gauge needle through the side of the tube. The ethidium bromide was removed by extracting the DNA three or four times with equal volumes of cold *n*-butanol. The DNA was then dialyzed overnight at 4°C in 20 mM Tris (pH 7.5)–0.1 mM EDTA and stored at 4°C.

**Transformation.** The transformation procedure was that of V. Hershfield and is a modification of that described by Cohen et al. (10).

The strain to be transformed was grown to Klett 50 ( $1 \times 10^8$  to  $2 \times 10^8$  cells per ml) in 20 ml of L broth, plus antibiotics if appropriate. The culture was chilled on ice before harvesting of the cells by centrifugation. The cells were then washed once with 10 mM NaCl, suspended in 10 ml of 30 mM  $\text{CaCl}_2$  (pH 7.2 to 7.4), and incubated on ice for 20 min. Finally, the cells were centrifuged and suspended in 1 ml of 100 mM  $\text{CaCl}_2$  (pH 7.2 to 7.4).

Samples consisted of 200  $\mu\text{l}$  of cells in 100 mM  $\text{CaCl}_2$  and 100  $\mu\text{l}$  of DNA in TEN buffer (20 mM Tris [pH 8.0], 1 mM EDTA, 20 mM NaCl). Controls consisted of 200  $\mu\text{l}$  of cells and 100  $\mu\text{l}$  of TEN with no DNA. The samples were incubated on ice for 60 min and then heated in a 42°C water bath for 2 min. Warm (37°C) L broth (2.7 ml) was added to each sample, and the tubes were incubated for 1.5 to 2 h at 37°C with shaking to allow expression. Antibiotic-resistant transformants were selected by plating on L plates containing the appropriate antibiotic(s).

**Agarose gel electrophoresis.** Electrophoresis was carried out on slab gels (10 by 13.5 by 0.6 cm) of 0.7% agarose in Tris-borate buffer (10.8 g of Tris base, 0.93 g of disodium EDTA, and 5.5 g of boric acid per liter of water; pH 8.0 to 8.3) as described by Hershfield et al. (25). Electrophoresis was performed at 100 to 120 V (50 to 65 mA) for about 4 h, in which time the bromophenol blue tracking dye used migrated to the bottom of the gel.

Resolution of the cointegrate plasmid bands was determined to be greatest when the total time for electrophoresis was relatively short (3 to 4 h). Attempts to improve resolution by substantially increasing the time for electrophoresis obscured the distinction between the cointegrate and the large plasmid, pNC21.

**EcoRI digestion.** Digestion of DNA by endo R-EcoRI was carried out as described by Hershfield et al. (25). Conditions for the digestion of DNA by the other restriction endonucleases used were as described by Graham et al. (18).

**Electron microscopy.** Purified pNC12 plasmid DNA was denatured with 0.1 M NaOH and neutralized with Tris-hydrochloride by the methods of Davis et al. (12). Renaturation, in the presence of 50% formamide, was allowed to proceed for only a few minutes at room temperature so that only intramolecular reannealing might occur. The renatured-DNA solution, containing approximately 1  $\mu\text{g}$  of DNA per ml, was made 0.1 mg/ml in cytochrome *c* and spread onto a

hypophase of 0.01 M Tris (pH 8.5) plus 40% formamide. Samples of the monolayer were picked up onto collodion-coated, 400-mesh, copper, electron microscope grids, stained with 0.004% aqueous uranyl acetate, and rotary shadowed with tungsten (44). Observations were made with a Siemens Elmiskop I electron microscope at a nominal magnification of  $\times 20,000$ .

## RESULTS

**Transmission of deletion mutant F-prime plasmids.** Guyer and Clark (21) isolated two types of nonconjugative deletion *FargG*<sup>+</sup> plasmids. The first type carried a deletion of some of the *tra* genes and was transmissible at high frequency in the presence of a *tra*<sup>+</sup> *Flac*<sup>+</sup> plasmid. One such plasmid is pJC59 which lacks *traD*, *traS*, *traH*, and *traF* activities; *traI* activity was not tested (Fig. 1). The second type of deletion had lost all detectable *tra* gene activity, and its *argG*<sup>+</sup> gene was transmissible only at very low frequency in the presence of a *tra*<sup>+</sup> *Flac*<sup>+</sup> plasmid. A representative of this type is pJC61, a 188-kb plasmid which carries a deletion of 61% of F extending clockwise on the standard map of F from 52.2 to 16.3 kb (22) and a substitution of 152 kb of bacterial DNA which includes *argG*<sup>+</sup> (Fig. 1). The first class was hypothesized to have retained the origin of conjugational transfer, *oriT*, and the second was hypothesized to have lost it (21). Since *oriT* is by definition a sequence required for mobilization, transfer, and possibly replication, the first class is expected to be transmissible whereas the second class is not.

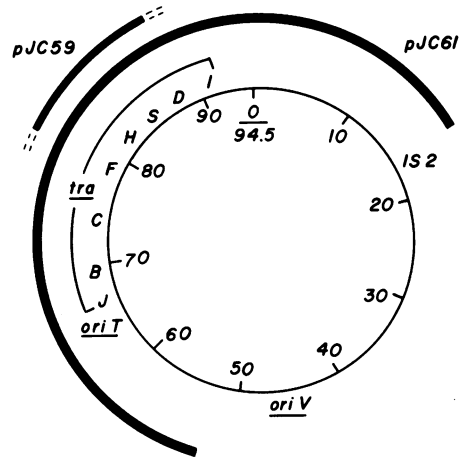


FIG. 1. Sequences of *F* deleted in *FargG*<sup>+</sup> plasmids pJC59 and pJC61. Map of *F* showing the kilobase coordinates from 0 to 94.5 and a partial genetic map. The inner and outer heavy lines span the sequences deleted in pJC61 and pJC59, respectively (21). The exact endpoints of the deletion in pJC59 have not been determined. IS, Insertion sequence; *tra*, transfer; *oriV*, origin of vegetative replication; *oriT*, origin of conjugational transfer.

The Guyer and Clark (21) experiments determining the transmissibility of pJC59 and pJC61 were performed with *recA56* transient heterozygotes to prevent high-frequency recombination of the homologous conjugative *Flac* and nonconjugative *Farg* plasmids and to circumvent the problem of incompatibility. In the experiments described here, we used as the conjugative plasmid pNC21, a deletion derivative of R1-19 which determines resistance to chloramphenicol and which is compatible with F. This allowed us to measure the transmission of the *FargG<sup>+</sup>* plasmids from stable strains carrying both the conjugative and nonconjugative plasmids. Like *Flac<sup>+</sup>*, pNC21 was able to promote high-frequency transmission of pJC59 but not of pJC61 in a *recA56* genetic background (Table 4).

We next investigated the nature of the plasmids inherited by *Arg<sup>+</sup>* *Cm<sup>r</sup>* transconjugants derived from the pJC59/pNC21 and pJC61/pNC21

donors (JC11616 and JC10862, respectively). We first made use of the differential sensitivity of the F and R1 replication-segregation systems to inhibition by acridine orange (41) to test the independence of the F *argG<sup>+</sup>* gene and the pNC21 *cat<sup>+</sup>* gene in the donor and progeny strains. Growth of the pJC59/pNC21 and pJC61/pNC21 donor strains in the presence of acridine orange led to loss of the *argG<sup>+</sup>* F-prime plasmid from 98% or more of the cells and loss of the pNC21 *cat<sup>+</sup>* gene from less than 2% of the cells. Two progeny (JC11637 and JC11638) derived from the pJC59/pNC21 donor showed the same response to acridine orange, indicating that the *argG<sup>+</sup>* and *cat<sup>+</sup>* genes had most likely been inherited on separate plasmids. The results with the progeny (JC11641 and JC11642) of the pJC61/pNC21 donor were different in that *argG<sup>+</sup>* was quite resistant to acridine treatment, with loss of the *Arg<sup>+</sup>* phenotype from less than 2% of the cells. This would have been expected if pJC61 had integrated into pNC21 and the *argG<sup>+</sup>* and *cat<sup>+</sup>* genes had been transferred as part of a single acridine orange-resistant plasmid cointegrate.

The presence of such a cointegrate plasmid was confirmed in four of the progeny of the pJC61/pNC21 donor (JC10862) by examining plasmid DNA by agarose gel electrophoresis. The results for two of the progeny are shown in Fig. 2b. The first progeny strain (JC11641) showed two plasmid bands, one with the electrophoretic mobility of pNC21 and one with lower mobility (i.e., higher molecular weight) than

TABLE 4. Frequencies of pNC21-promoted transmission of pJC59 and pJC61 to recipient JC7214

Donor strain	Plasmid in donor strain	Progeny/100 donors		<i>Arg<sup>+</sup></i> progeny/ <i>Cm<sup>r</sup></i> progeny
		<i>Arg<sup>+</sup></i> [ <i>Leu<sup>+</sup></i> <i>Ura<sup>+</sup></i> ]	<i>Cm<sup>r</sup></i> [ <i>Leu<sup>+</sup></i> <i>Ura<sup>+</sup></i> ]	
JC7247	pJC59	$<5 \times 10^{-5}$		
JC11616	pJC59 pNC21	63	180	$4 \times 10^{-1}$
JC7249	pJC61	$<6 \times 10^{-5}$		
JC10862	pJC61 pNC21	$9 \times 10^{-4}$	3	$3 \times 10^{-4}$

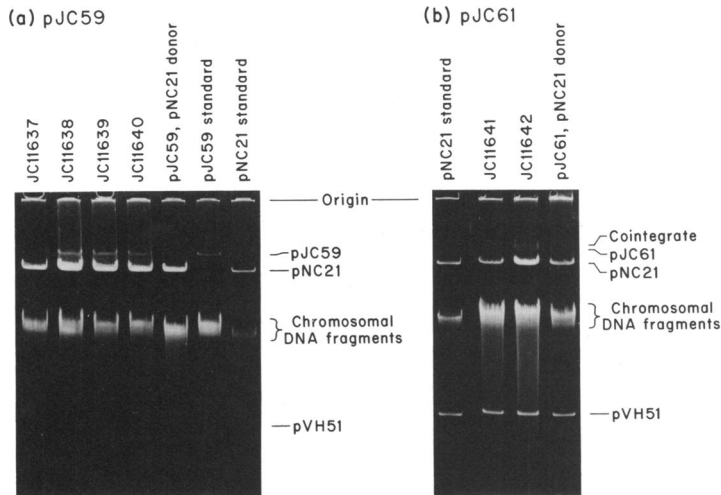


FIG. 2. Agarose gel patterns of plasmid DNA from strains involved in transmission of pJC59 and pJC61. Plasmid DNA was prepared by the method of Meyers et al. (32) and analyzed by electrophoresis on 0.7% agarose slab gels. *EcoRI*-treated pVH51 DNA (a 3.3-kb plasmid with one *EcoRI* site [24]) was added to each sample as an *R<sub>f</sub>* standard. (a) DNA from a donor carrying pJC59 and pNC21 and from four transconjugants inheriting *argG<sup>+</sup>* and chloramphenicol resistance. (b) DNA from a donor carrying pJC61 and pNC21 and from two transconjugants inheriting *argG<sup>+</sup>* and chloramphenicol resistance.

pJC61. The second progeny (JC11642) showed plasmid bands with the same mobility as those of the first progeny and, in addition, showed one with the mobility of pJC61. DNA from the other two progeny also contained three bands with mobilities identical to those of the second progeny strain bands (data not shown). By contrast all four progeny from the pJC59 × pNC21 cross (JC11637 × JC11640) contained only two DNA bands corresponding to the original two plasmids (Fig. 2a); a band with mobility lower than that of the pJC59 band was not seen.

These results lead to the conclusion that high-frequency *argG*<sup>+</sup> transmission from a pJC59/pNC21 donor was correlated with the inheritance of the original *FargG*<sup>+</sup> plasmid (pJC59) independent of the inheritance of pNC21. This would be expected if nonconjugative *FargG*<sup>+</sup> plasmid pJC59 carried an *oriT* site which rendered it capable of being mobilized as an entity separate from the conjugative plasmid pNC21. Very-low-frequency *argG*<sup>+</sup> transmission from the pJC61/pNC21 donor, by contrast, seemed to be correlated with the inheritance of a new plasmid whose replication was insensitive to acridine orange treatment, as was that of pNC21. Therefore, transmission of this *oriT* mutant appeared to occur by a different mechanism and not simply by low-efficiency transfer initiating at a secondary *oriT* site. We thought that the new plasmid was likely to be a cointegrate of pJC61 and pNC21, but since it was difficult to estimate the size of such a large plasmid and to investigate its structure, we changed the focus of our studies to the transmission of pML31. This is a smaller plasmid which was constructed in vitro from the replicative *EcoRI* fragment 5 (f5) of F and a DNA fragment determining resistance to the antibiotic kanamycin (28). It resembles pJC61 in that it carries *EcoRI* fragment 5 of F and lacks fragment 6. Because pML31 lacks all of the *tra* genes, it is nonconjugative. Because it lacks fragment 6 containing *oriT*, we suspected that it would be nontransmissible.

**Transmission of pML31: Nature of progeny plasmids.** The transmission frequency of the *kan*<sup>+</sup> gene of pML31 from a donor carrying pML31 and pNC21 (JC10898) was measured. The results of two separate experiments are shown in Table 5. The transmission of *kan*<sup>+</sup> was approximately the same as that of the *arg*<sup>+</sup> gene of pJC61 relative to the transmission of the *cat*<sup>+</sup> gene of pNC21 (Table 4). To determine whether *kan*<sup>+</sup> was capable of independent inheritance, like the *arg*<sup>+</sup> gene of pJC59, or whether its inheritance was dependent on formation of a recombinant plasmid with pNC21, as with pJC61, we prepared plasmid DNA, by the Meyers method, from the pML31/pNC21 donor strain (JC10898) and from four of the Km<sup>r</sup> transconjugants derived from it in experiment 1. Agarose gel analysis of the DNA from the original donor showed bands corresponding to those of pML31 and pNC21 (Fig. 3). The transconjugants, however, each contained three plasmid bands: one which appeared to be pNC21, one which was larger than pNC21, and one which was larger than pML31 but smaller than pNC21. Plasmid DNA from two such transconjugants, JC11625 and JC11626, is shown in Fig. 3. Plasmid DNA analyses of four transconjugants from experiment 2, including JC11130 and JC11131, showed that all four similarly had three bands.

To characterize the new small plasmid, DNA was prepared from JC11625 and JC11626 by the method of Clewell and Helinski (9). This plasmid DNA, purified by cesium chloride-ethidium bromide density equilibrium centrifugation, was used to transform *recA56* strain JC7199 to kanamycin resistance. To make certain that pNC21, or a derivative, was not being inherited by congression (i.e., by uptake of two independent DNA molecules), the Km<sup>r</sup> transformants were tested for sensitivity to chloramphenicol and ampicillin. None of 50 Km<sup>r</sup> transformants derived from JC11625 DNA was Cm<sup>r</sup>, and only 4 of the 50 derived from JC11626 were Cm<sup>r</sup>. By contrast, all of the Km<sup>r</sup> transformants had in-

TABLE 5. Relative transmission frequencies of *kan*<sup>+</sup> and *cat*<sup>+</sup> by a pML31/pNC21 strain and its transconjugant progeny

Comment	Donor	Recipient	Km <sup>r</sup> [ <i>x</i> <sup>+</sup> <i>y</i> <sup>+</sup> ] <sup>a</sup> progeny/100 do- nors	Cm <sup>r</sup> [ <i>x</i> <sup>+</sup> <i>y</i> <sup>+</sup> ] <sup>a</sup> progeny/100 do- nors	Km <sup>r</sup> progeny/ Cm <sup>r</sup> progeny
Expt 1	JC10898	JC7199	5 × 10 <sup>-3</sup>	25	2 × 10 <sup>-4</sup>
	JC11625	JC7532	14	19	7 × 10 <sup>-1</sup>
	JC11626	JC7532	7	9	8 × 10 <sup>-1</sup>
Expt 2	JC10898	JC7199	1 × 10 <sup>-3</sup>	42	3 × 10 <sup>-4</sup>
	JC11130	JC7532	9 × 10 <sup>-1</sup>	1	8 × 10 <sup>-1</sup>
	JC11131	JC7532	1	1	9 × 10 <sup>-1</sup>
Transformant	JC11128	JC7532	2 × 10 <sup>-4</sup>	2	1 × 10 <sup>-4</sup>
Transformant	JC11129	JC7532	3 × 10 <sup>-1</sup>	6 × 10 <sup>-1</sup>	5 × 10 <sup>-1</sup>

<sup>a</sup> *x*<sup>+</sup> *y*<sup>+</sup> is Pro<sup>+</sup> TL<sup>+</sup> for matings with JC7199 as the recipient and Arg<sup>+</sup> for matings with JC7532 as the recipient.

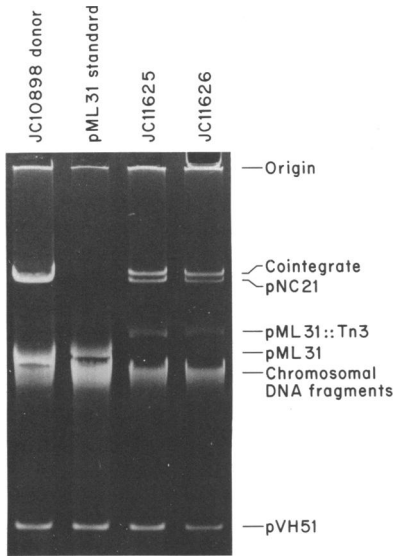


FIG. 3. Plasmids from *Km<sup>r</sup>* progeny derived from a *pML31/pNC21* donor. Conditions were as described in the legend to Fig. 1.

herited ampicillin resistance. Since ampicillin resistance was originally carried by a region of *pNC21* which has been identified as insertion element *Tn3*, we suspected that transposition of *Tn3* had occurred to form a *pML31::Tn3* mutant plasmid (11, 26, 27).

To confirm this, plasmid DNA was isolated from one of the *Km<sup>r</sup> Cm<sup>s</sup> Ap<sup>r</sup>* transformants constructed by using DNA from *JC11625* and was analyzed by agarose gel electrophoresis. Figure 4 shows the untreated and *EcoRI*-treated samples of this plasmid, designated *pNC12*, compared with untreated and *EcoRI*-treated *pML31* DNA. The *EcoRI*-treated sample of the DNA from the transformant showed two bands, one with the same mobility as that of the *kan* fragment of *pML31* and one with a mobility lower than that of *f5*. We estimated the size of this fragment to be 13.2 kb based on its mobility relative to those of the *EcoRI* bands of *R1-19*. This size correlated well with that expected for a DNA fragment composed of *f5* (9.0 kb) and *Tn3* (4.8 kb). The addition of no new *EcoRI* cleavage sites to the mutant plasmid is consistent with this structure, since no *EcoRI* sites are contained in *Tn3* (26). Furthermore, the inserted DNA introduced one additional *BamHI* site and three additional *PstI* sites into *pML31*, which is a characteristic of *Tn3* (Fig. 5). Finally, electron microscope observation of the new plasmid showed the stem-loop structure typical of *Tn3* (Fig. 6). An analogous analysis of the mutant plasmid designated *pNC13* in transconjugant *JC11626* indicated that it too was due to *Tn3*

transposition from *pNC21* to *f5* of *F* in *pML31*.

The precise sites of *Tn3* insertions in *pNC12* and *pNC13* were determined by digesting the two plasmid DNAs with restriction endonucleases *BamHI*, *HindIII*, and *PstI*. Endonuclease *BamHI* cleaved *pML31* at two sites to produce two fragments, one of 13.9 kb and another of 2.4 kb (29). A comparison of the fragments produced by *BamHI* digestion of *pML31*, *pNC12*, and *pNC13* (Fig. 5) demonstrated that the *Tn3* insertion occurred in the 2.4-kb fragment, producing *pNC12*, and in the 13.9-kb fragment, producing *pNC13*. Since *Tn3* itself contains a single *BamHI* site, its insertion results in the production of two new fragments whose sizes can be used to determine the position of the inserted *BamHI* site. The *BamHI* digest of *pNC12* showed two new fragments of 1.5 and 5.7 kb in length. Since the *BamHI* site in *Tn3* has been mapped at a position 1.4 kb from one end of *Tn3* (23), we inferred that the *Tn3* insertion in *pNC12* occurred within the 2.4-kb *BamHI* fragment of *pML31*, at a site 0.1 kb from one end. Digestion of *pNC12* DNA with *HindIII*, which cleaves *pML31* into two unequal-sized fragments (29),

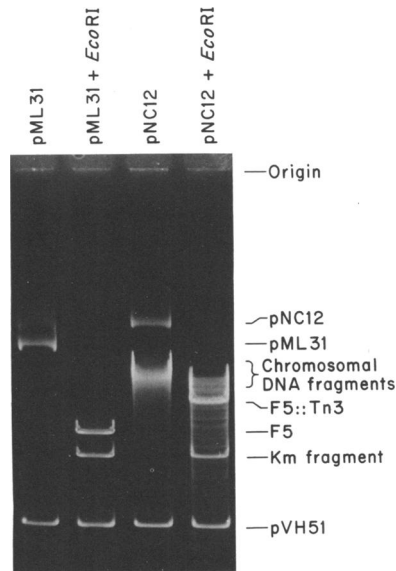


FIG. 4. *EcoRI* analysis of *pNC12* from *JC11625*. *pML31* DNA was prepared from *CR34(pML31)* by the method of Clewell and Helinski (9). *pNC12* DNA was prepared, by the method of Meyers et al. (32), from *JC13403*, a *Km<sup>r</sup>* transformant made by using total plasmid DNA prepared by the Meyers method from *JC11625*, and dialyzed against 20 mM Tris (pH 7.5)-0.1 mM EDTA before *EcoRI* treatment. Untreated and *EcoRI*-treated *pML31* and *pNC12* DNAs were analyzed on an 0.7% agarose slab gel. *EcoRI*-treated *pVH51* DNA was added to each sample as an *R<sub>I</sub>* standard.

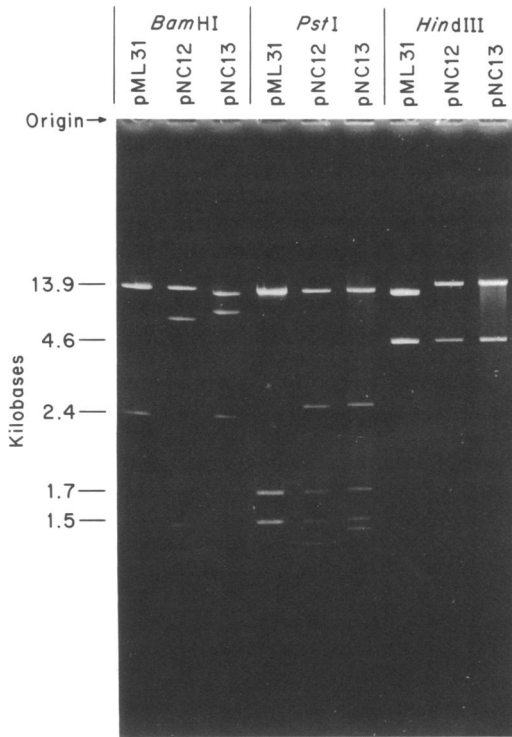


FIG. 5. Restriction enzyme analysis of plasmids pNC12 and pNC13. Plasmids pML31, pNC12, and pNC13, purified by the method of Meyers et al. (32), were digested by the restriction endonucleases indicated and analyzed by electrophoresis on a 0.7% agarose slab gel.

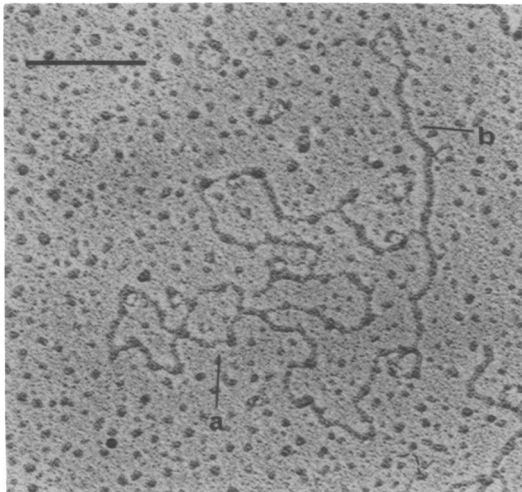


FIG. 6. Electron micrograph of pNC12 DNA. Purified pNC12 DNA was denatured, rapidly renatured to permit intrastrand reannealing, and prepared for electron microscopy as described in the text. (a) Stem-loop structure due to Tn3; (b) stem-loop structure due to the fragment of pML31 which carries the gene for kanamycin resistance (35). Bar, 0.25  $\mu$ m.

showed that the Tn3 insertion was in the larger *Hind*III fragment of pML31. We concluded that the Tn3 insertion into pML31 that formed pNC12 occurred at the 43.1-kb coordinate of *Eco*RI fragment 5 (as measured on the standard map of F) and has the orientation shown in Fig. 7.

The sizes of the two new *Bam*HI fragments resulting from pNC13 digestion indicated that the Tn3 insertion occurred at either the 46.0- or the 48.0-kb coordinate, depending on the orientation of Tn3. To distinguish which of these two possible sites was correct, we digested pNC13 with restriction endonuclease *Pst*I. This enzyme cleaved pML31 into four fragments, three of which could be easily seen on agarose gels (Fig. 5) (15). The smallest visible fragment was defined by coordinates 45.7 and 47.2, and included one of the possible Tn3 insertion sites. Since that fragment was also produced by *Pst*I digestion of pNC13, we concluded that the Tn3 insertion in pML31 that resulted in pNC13 occurred at the 48.0-kb coordinate of the f5 fragment and had the orientation shown in Fig. 7.

We next investigated the nature of the plasmids larger than pNC21 by restriction endonuclease digestion of plasmid DNA prepared by

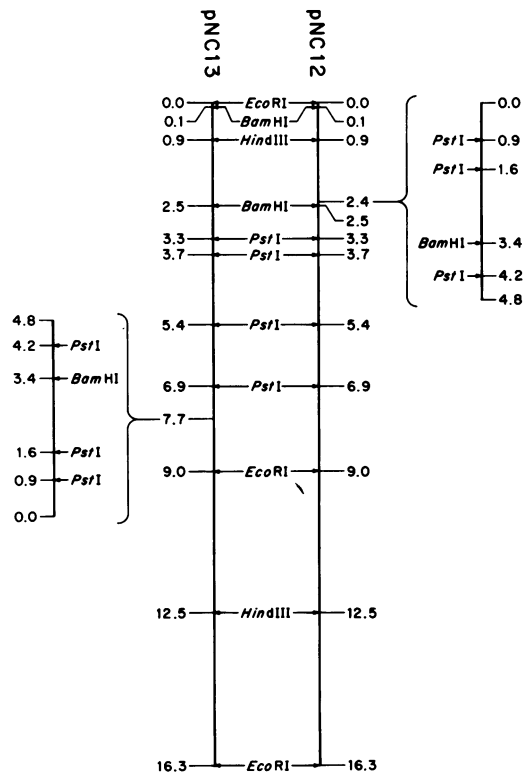


FIG. 7. Restriction map of pNC12 and pNC13, indicating the Tn3 insertion sites in pML31.



the method of Meyers et al. (32) from transconjugants JC11625 and JC11626. DNA from these two strains contained a mixture of three plasmids (pNC21, pNC12, or pNC13) and a plasmid larger than pNC21. Therefore, the bands derived from restriction endonuclease digestion of the new large plasmid would be superimposed on the bands derived from pNC21 and pNC12 or pNC13. The agarose gel analyses of these DNA preparations treated with restriction endonucleases *EcoRI*, *BamHI*, and *HindIII* are shown in Fig. 8.

The *EcoRI* digest of plasmid DNA from JC11625 showed one new band of about 10.2 kb which could not be attributed to either pNC21 or pNC12. The digest also showed an increased intensity of *EcoRI* fragment 3 of pNC21 and a decreased intensity of fragment 4. The *BamHI* digest of JC11625 plasmid DNA showed no bands other than those expected from pNC21 and pNC12. The *HindIII* digest of that same DNA preparation, however, showed two new bands whose sizes we judged to be approximately 9.5 and 18 kb and which we attributed to the new large plasmid in JC11625. These data, in particular the absence of any new bands after digestion of JC11625 plasmid DNA with *BamHI*, led us to propose that the new large plasmid in JC11625 was a cointegrate of pNC21

and pNC12 whose structure was such that the junctions of the two plasmids was marked by two Tn3 sequences arranged in the same orientation. Our model for the putative cointegrate in JC11625, designated pNC14, predicted that there would be no new *BamHI* fragment, two new *EcoRI* fragments (10.35 and 13.40 kb), and two new *HindIII* fragments (8.95 and 21.95 kb). These predicted fragment sizes are in reasonably good agreement with the new fragments we observed using the three restriction endonucleases. The new 13.40-kb *EcoRI* band was not resolved presumably because of its proximity to the 12.9-kb R3 fragment derived from pNC21.

Similarly, we hypothesized that the new large plasmid (pNC15) found in transconjugant JC11626 is a cointegrate of pNC21 and pNC13 whose junctions are similarly defined by Tn3 sequences in the same orientation. The predicted *EcoRI* digestion products of pNC15 include two unique fragments of 9.1 and 14.8 kb, which could not be distinguished easily on our gels from *EcoRI* fragment R4 of pNC21 (10.0 kb) and the larger *EcoRI* fragment from pNC13 (13.8 kb). Our hypothetical pNC15 structure should yield no new *BamHI* fragments and is consistent with our observation that *BamHI* digestion of JC11626 produced no bands unique to pNC15. Finally, a *HindIII* digest of pNC15

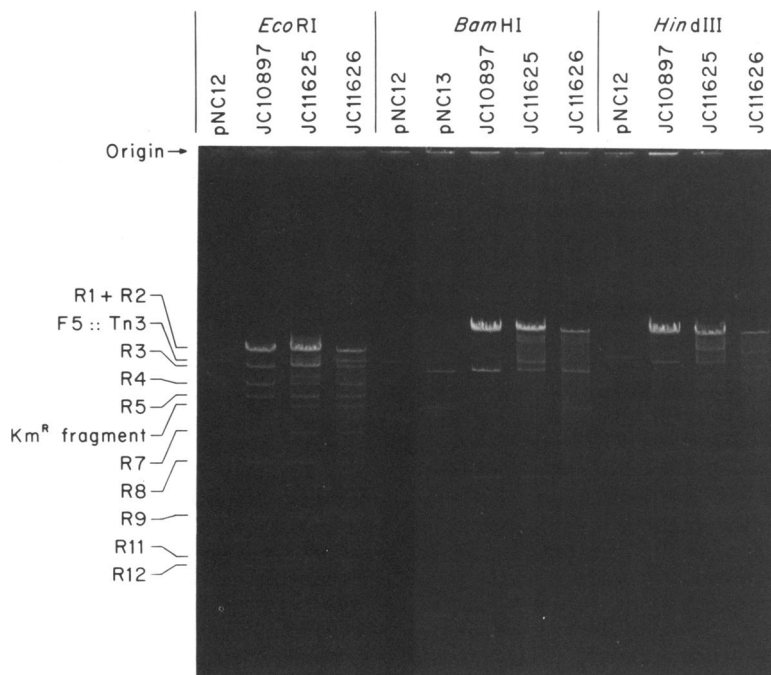


FIG. 8. Restriction enzyme analysis of total plasmid DNA from transconjugants JC11625 and JC11626. Plasmid DNAs purified by the method of Meyers et al. (32) from strains JC10897, JC11625, and JC11626 were treated with the indicated restriction endonucleases and analyzed by electrophoresis on 0.7% agarose gels. The identities and sizes of the *EcoRI* fragments of pNC21 (JC10897) are as reported by Blohm and Goebel (5).

DNA showed two new bands whose sizes (13.0 and 16.8 kb) were in reasonably good agreement with those predicted (12.1 and 19.9 kb) for the cointegrate structure proposed for pNC15.

**Transmissibility of *kan*<sup>+</sup> is associated with the presence of pNC14 and pNC15.** If plasmids pNC14 and pNC15 are cointegrates of pNC21 and pML31::Tn3, then the *kan*<sup>+</sup> gene of pML31 should be transmissible conjugationally at approximately the same frequency as the *cat*<sup>+</sup> gene of pNC21. To test this, transconjugants JC11625, JC11626, JC11130, and JC11113 were crossed with JC7532, a *recA56* recipient; Km<sup>r</sup> [Arg<sup>+</sup>] and Cm<sup>r</sup> [Arg<sup>+</sup>] transconjugants were selected separately. The data (Table 5) show that the *kan*<sup>+</sup> and *cat*<sup>+</sup> genes were transmitted to JC7532 at about equal frequencies. This contrasts with the relative transmission frequency of  $2 \times 10^{-4}$  for these genes shown in the original cross of JC10898 with JC7199. We tentatively concluded that the *kan*<sup>+</sup> gene had been inherited in a high-frequency transmissible state.

The tentativeness was based on the fact that the population of cells called JC11625 and JC11626 contained three plasmid species, only one of which was pNC14 or pNC15, respectively. If pNC14 and pNC15 are indeed cointegrate plasmids, we would expect them to be incompatible with their constituent plasmids, as was shown by Cabello et al. (6) for a pSC101-ColE1 cointegrate. Therefore, we believe that the presence of three plasmid bands in the DNA prepared from JC11625 and JC11626 indicates that these two strains consist of a mixed population of cells, some of which carry only the cointegrate and some of which carry the two autonomous plasmids derived from the breakdown of the cointegrate in some of the cells descended from the original transconjugant. To separate these two large plasmids from the smaller plasmids, plasmid DNA, prepared from JC11625 and JC11626 by the method of Clewell and Helinski (9), was used to transform JC7199 to kanamycin resistance. The unselected coinherence of ampicillin resistance and chloramphenicol resistance was scored. Owing to the presence of Tn3 on pNC12 and pNC13, 50 Km<sup>r</sup> transformants derived from JC11625 DNA were all Ap<sup>r</sup>, as were 50 derived from JC11626 DNA. Four of the 50 Km<sup>r</sup> Ap<sup>r</sup> transformants produced by DNA from JC11626 were also Cm<sup>r</sup>. Total plasmid DNA from two of these was prepared by the Meyers method and examined by agarose gel electrophoresis. The results (Fig. 9) showed that DNA from one of these transformants, JC11128, contained two plasmid bands with the mobilities characteristic of the pNC12 (pML31::Tn3) and pNC21 bands. DNA from the other transformant, JC11129, contained one main band with the

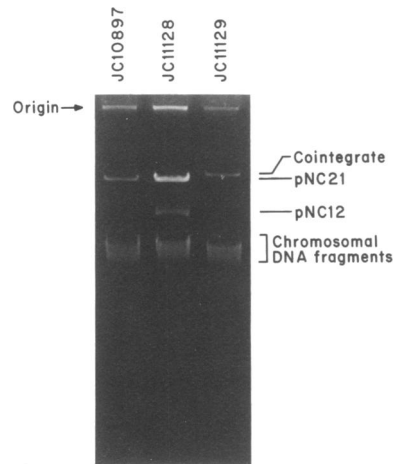


FIG. 9. Agarose gel analysis of plasmid DNA from transformants JC11128 and JC11129. Plasmid DNAs were purified by the method of Meyers et al. (32) from strains JC10897, JC11128, and JC11129 and analyzed by electrophoresis on 0.7% agarose gels.

mobility of the largest plasmid (pNC15) and two faint bands in the positions of pNC21 and pNC12. Conjugational transmission of *kan*<sup>+</sup> from JC11129, containing the suspected cointegrate, was high, whereas that from JC11128, which carried the two plasmids separately, was low (Table 5). This also supports the hypothesis that the cointegrate plasmid pNC15 represents the highly transmissible form of the *kan*<sup>+</sup> gene. Although we did not similarly confirm that pNC14 is a highly transmissible form of the *kan*<sup>+</sup> genes, we assume that it is.

**Frequency of Tn3 transposition mutants in a pNC15 strain.** Since other investigators (16) had reported that cointegrate molecules could only be detected if the process was mediated by certain transposition-defective mutants of Tn3, we measured the ability of the Tn3 sequences on pNC15, a relatively stable cointegrate, to transpose to another replicon, pSC101. Our assay for transposition was based on the resistance of cells carrying pSC101::Tn3 to high levels of ampicillin because of the increased number of *bla* genes available in those cells and will be detailed elsewhere (J. A. Nowak and A. J. Clark, in preparation). Our results indicated that the frequency of Tn3 transposition mutants of pSC101 in the presence of Tn3 donor pNC15 was  $5.8 \times 10^{-2}$  (mutants per total cells) and was essentially the same as the frequency observed for Tn3 transposition mutants of pSC101 in the presence of pNC21. Hence, it appears that the Tn3 sequences involved in formation of pNC15 were not altered in their ability to transpose.

## DISCUSSION

We determined the transmission frequencies of two nonconjugative *FargG*<sup>+</sup> plasmids by using pNC21 as the coresident conjugative plasmid. In the case of pJC59, which retained *oriT*, the sequence at which F plasmids are presumed to be mobilized for transfer (1, 11), we observed that the transmission of *argG*<sup>+</sup> was nearly as high as that of the *cat*<sup>+</sup> genes of pNC21. In addition, transconjugant progeny inheriting both *argG*<sup>+</sup> and *cat*<sup>+</sup> carried plasmids having electrophoretic mobilities of the original plasmids. pJC59 can therefore be classified as transmissible since it carries the genes needed to effect the mobilization, transfer, and replication of its own DNA. In the case of pJC61, which was deleted for *EcoRI* fragment 6 and therefore lacked *oriT*, the transmission frequency of *argG*<sup>+</sup> was only 10<sup>-4</sup> as great as the transmission of *cat*<sup>+</sup>. Transconjugants inheriting *argG*<sup>+</sup> and *cat*<sup>+</sup> showed three plasmid DNA bands on agarose gels. The smaller two were indistinguishable in mobility from the original plasmids, pNC21 and pJC61. (Although we observed no change in mobility of pJC61, the transconjugants most likely carried pJC61::Tn3 by analogy with the pML31 results. The addition of a 4.8-kb Tn3 sequence to the 188-kb pJC61 DNA would not cause a detectable change in mobility.) The largest plasmid had a mobility consistent with its being a cointegrate of pNC21 and pJC61. We therefore concluded that pJC61 was nontransmissible since it was incapable of effecting its own mobilization, transfer, and replication as an independent plasmid.

In comparing the transmission of pJC59 and pJC61, we discovered that the low-frequency transmission of the *oriT* mutant, pJC61, was not simply low-efficiency mobilization, perhaps from a secondary *oriT* site but, rather, was brought about by an entirely different mechanism which was dependent upon recombination between the conjugative and nonconjugative plasmids.

Since the donor strain carrying pJC61 and pNC21 was *recA56*, we did not expect recombination between homologous sequences to be the basis for the hypothesized cointegration of the two plasmids. To investigate this basis, we used pML31, a nonconjugative plasmid much smaller than pJC61 but related to it in that both plasmids were deleted for *EcoRI* fragment 6 and retained the replication region of F. The pML31 results were parallel to those with pJC61 as far as the low-frequency transmission of the pML31 *kan*<sup>+</sup> genes relative to *cat*<sup>+</sup> transmission and the appearance of a new large plasmid presumed to be a cointegrate. In addition, we found that the Km<sup>r</sup> Cm<sup>r</sup> transconjugants no longer carried

pML31 but, rather, a new small plasmid identified as pML31::Tn3. On the basis of restriction enzyme analyses, we propose that the new large plasmid is a cointegrate of pML31::Tn3 and pNC21 in which there are two copies of Tn3 in direct repeat on either side of pML31.

The cointegrate and the insertion mutant forms of pML31 provide a clue to the nature of the *recA*-independent recombination events permitting transmission of pML31 genes. Two hypotheses are testable: (i) transposition of Tn3 to pML31 occurs, and then the separate pML31::Tn3 and pNC21 plasmids recombine by a Tn3-specific event to form the cointegrate; (ii) transposition of Tn3 to pML31 involves an intermediate whose structure is that of the cointegrate with two copies of Tn3 and whose components can subsequently separate by a Tn3-specific event. We found the cointegrate to be a high-frequency transmissible form of the *kan*<sup>+</sup> gene, which is consistent with both hypotheses. We also found that *recA56* strains carrying both pML31::Tn3 and pNC21 were very-low-frequency donors of *kan*<sup>+</sup>, an observation which is inconsistent with the first hypothesis for the following reason. The frequency of *kan*<sup>+</sup> transfer should reflect the frequency of the event(s) required for transfer. If transposition and subsequent recombination are both required, as in the first hypothesis, the transfer frequency would equal the product of the frequency of transposition and the frequency of Tn3-specific recombination. We would therefore expect to see higher *kan*<sup>+</sup> transmission from a pML31::Tn3/pNC21 donor, where transposition had already taken place, than from a pML31/pNC21 donor. Presumably, the frequency of transfer of *kan*<sup>+</sup> from the pML31::Tn3/pNC21 donor represents the frequency of Tn3-specific *recA*-independent recombination between the separate plasmids to generate the cointegrate. However, since this frequency is as low as *kan*<sup>+</sup> transmission from the pML31/pNC21 donor, the cointegrate plasmid does not appear to be formed as a consequence of these two low-frequency events.

Thus, we are led to hypothesize that cointegrate formation is a normal part of Tn3 transposition from one plasmid to another and that such cointegrates contain two copies of Tn3 in direct repeat at each end of the plasmid to which transposition is occurring. Cointegrate molecules with the same basic structure have been reported by a number of other investigators and have involved transposons Tn3 (16, 23, 27, 31), insertion sequence IS1 (30), and bacteriophage Mu (39). Gill et al. (16) and Meyer et al. (31) have reported cointegrate formation mediated by mutants of Tn3 and Tn5, respectively. These authors have proposed that *recA*-independent

Tn3 sequence-specific recombination is one route for separating cointegrate plasmids into their component parts to complete transposition. Our results are consistent with this proposal in that they show breakdown of the cointegrate in *recA56* cells. Our results are inconsistent with the implied hypothesis that cointegrate transposition intermediates are undetectable unless one uses mutant derivatives of Tn3.

Although we believe that the cointegrates we observed are formed as intermediates in transposition, we feel that cointegrates need not be obligatory intermediates in all transposition events. The mechanisms for transposition proposed by Grindley and Sherratt (19) and Shapiro (34) may involve a concerted set of reactions, making cointegrates an obligatory intermediate. However, if the reactions are not concerted and if certain of the intermediates are available as substrates for alternative reactions, then cointegrates may represent only one pathway of transposition. The frequency of transmission of the genes of a non-mobilizable plasmid permitted by transposition-mediated recombination would be expected to vary with the frequency and stability of the cointegrate molecules formed.

In summary, we observed that the low-frequency transmission of plasmids lacking the *oriT* site is dependent upon a recombination event between the coresident conjugative and nonconjugative plasmids. This is an example of the process called "conduction" by Clark and Adelbert (7) to distinguish it from the process of donation (8), in which conjugative and nonconjugative plasmids are transmitted as separate entities. The recombination leading to conduction occurs during the process of the transposition of Tn3 from one plasmid to the other. The result is the formation of a cointegrate plasmid consisting of the conjugative and nonconjugative plasmids separated by direct repeats of the transposon and therefore capable of the conjugational transmission of the genes of both plasmids.

In the cases described here, the transposable sequence was present on the conjugative plasmid. This is similar to the situation described by Guyer (20), in which the gamma-delta sequence of F permitted *recA*-independent transmission of nonconjugative plasmid pBR322. It is also possible for a transposable sequence on the nonconjugative plasmid to facilitate cointegrate formation and, hence, conduction. This is presumably the situation studied by Goebel et al. (17), in which Tn3 was present on nonconjugative derivatives of R1. It should be noted that in such cases when the cointegrate separates into its components the original nonconjugative plasmid

will be regenerated and the conjugative plasmid will contain a new transposable element. If the size of the conjugative plasmid greatly exceeds that of the added element, it may be difficult to detect that the conjugative plasmid is actually a mutant.

The cointegrates that we observed were stable through the processes of mobilization, transfer, and replication although they did decay into their component plasmids at an easily detectable frequency. They may, therefore, provide an opportunity for studying intermediates in transposition and may be helpful in elucidating the steps in this process.

#### ACKNOWLEDGMENTS

This investigation was supported in part by Public Health Service grant AI 05371 from the National Institute of Allergy and Infectious Diseases and by grant NP-237 from the American Cancer Society to A.J.C. J.A.N. was supported by Public Health Service National Research Science Award AI 05880 from the National Institute of Allergy and Infectious Diseases. N.J.C. was supported in part by Public Health Service training grant GM 01389 from the National Institute of General Medical Sciences.

#### LITERATURE CITED

1. Achtman, M., R. A. Skurray, P. Thompson, R. Helmut, S. Hall, L. Beutin, and A. J. Clark. 1978. Assignment of *tra* cistrons to *EcoRI* fragments of F sex factor DNA. *J. Bacteriol.* **133**:1383-1392.
2. Bachmann, B. J. 1972. Pedigrees of some mutant strains of *Escherichia coli* K-12. *Bacteriol. Rev.* **36**:525-557.
3. Bachmann, B. J., K. B. Low, and A. L. Taylor. 1976. Recalibrated linkage map of *Escherichia coli* K-12. *Bacteriol. Rev.* **40**:116-167.
4. Beckwith, J. R., A. B. Pardee, R. Austrian, and F. Jacob. 1962. Coordination of the synthesis of the enzymes in the pyrimidine pathways of *E. coli*. *J. Mol. Biol.* **5**:618-634.
5. Blohm, D., and W. Goebel. 1978. Restriction map of the antibiotic resistance plasmid R1 *drd-19* and its derivatives *pKN 102* (R1 *drd-19B2*) and R1 *drd-16* for the enzymes *Bam*HI, *Hind*III, *Eco*RI and *Sal*I. *Mol. Gen. Genet.* **187**:119-127.
6. Cabello, F., K. Timmis, and S. N. Cohen. 1976. Replication control in a composite plasmid constructed by *in vitro* linkage of two distinct replicons. *Nature (London)* **259**:285-290.
7. Clark, A. J., and E. A. Adelberg. 1962. Bacterial conjugation. *Annu. Rev. Microbiol.* **16**:289-319.
8. Clark, A. J., and G. J. Warren. 1979. Conjugal transmission of plasmids. *Annu. Rev. Genet.* **13**:99-125.
9. Clewell, D. B., and D. R. Helinski. 1970. Properties of a supercoiled deoxyribonucleic acid-protein relaxation complex and strand specificity of the relaxation event. *Biochemistry* **9**:4428-4440.
10. Cohen, S. N., A. C. Y. Chang, and L. Hsu. 1972. Nonchromosomal antibiotic resistance in bacteria: genetic transformation in *Escherichia coli* by R-factor DNA. *Proc. Natl. Acad. Sci. U.S.A.* **69**:2110-2114.
11. Crisona, N., and A. J. Clark. 1977. Increase in conjugational transmission frequency of nonconjugative plasmids. *Science* **196**:186-187.
12. Davis, R. W., M. Simon, and N. Davidson. 1971. Electron microscope heteroduplex methods for mapping regions of base sequence homology in nucleic acids. *Methods Enzymol.* **21D**:413-428.
13. Eggertsson, G., and E. A. Adelberg. 1965. Map posi-

- tions and specificities of suppressor mutations in *Escherichia coli* K-12. *Genetics* **52**:319-340.
14. Falkinham, J. O., III, and A. J. Clark. 1974. Genetic analysis of a double male strain of *Escherichia coli* K-12. *Genetics* **78**:633-644.
  15. Figurski, D., R. Kolter, R. Meyer, M. Kahn, R. Eichenlaub, and D. R. Helinski. 1978. Replication regions of plasmids ColE1, F, R6K, and RK2, p. 105-109. In D. Schlessinger (ed.), *Microbiology*—1978. American Society for Microbiology, Washington, D.C.
  16. Gill, R., F. Heffron, G. Dougan, and S. Falkow. 1978. Analysis of sequences transposed by complementation of two classes of transposition-deficient mutants of Tn3. *J. Bacteriol.* **136**:742-756.
  17. Goebel, W., W. Lindennaier, F. Pfeifer, H. Schrempf, and B. Schelle. 1977. Transposition and insertion of intact, deleted, and enlarged ampicillin transposon Tn3 from mini-R1 (Rsc) plasmids into transfer factors. *Mol. Gen. Genet.* **157**:119-129.
  18. Graham, R. S., F. E. Young, and G. A. Wilson. 1977. Effect of site-specific endonuclease digestion on the *thyP3* gene of bacteriophage  $\phi$ 3T and the *thyP11* gene of bacteriophage  $\rho$ 11. *Gene* **1**:169-180.
  19. Grindley, N. D. F., and D. Sherratt. 1978. Sequence analysis at IS1 insertion sites: models for transposition. *Cold Spring Harbor Symp. Quant. Biol.* **43**:1257-1262.
  20. Guyer, M. S. 1978. The  $\gamma\delta$  sequence of F is an insertion sequence. *J. Mol. Biol.* **126**:347-365.
  21. Guyer, M. S., and A. J. Clark. 1976. *cis*-dominant, transfer-deficient mutants of the *Escherichia coli* K-12 F sex factor. *J. Bacteriol.* **125**:233-247.
  22. Guyer, M. S., N. Davidson, and A. J. Clark. 1977. Heteroduplex analysis of *tra* $\Delta$  F' plasmids and the mechanism of their formation. *J. Bacteriol.* **131**:970-980.
  23. Heffron, F., P. Bedinger, J. J. Champoux, and S. Falkow. 1977. Deletions affecting the transposition of an antibiotic resistance gene. *Proc. Natl. Acad. Sci. U.S.A.* **74**:702-706.
  24. Hershfield, V., H. W. Boyer, L. Chow, and D. R. Helinski. 1976. Characterization of a mini-ColE1 plasmid. *J. Bacteriol.* **126**:447-453.
  25. Hershfield, V., H. W. Boyer, C. Yanofsky, M. A. Lovett, and D. R. Helinski. 1974. Plasmid ColE1 as a molecular vehicle for cloning and amplification of DNA. *Proc. Natl. Acad. Sci. U.S.A.* **71**:3455-3459.
  26. Kopecko, D. J., J. Brevet, and S. N. Cohen. 1976. Involvement of multiple translocating DNA segments and recombinational hotspots in the structural evolution of bacterial plasmids. *J. Mol. Biol.* **108**:333-360.
  27. Kopecko, D. J., and S. N. Cohen. 1975. Site-specific *recA*-independent recombination between bacterial plasmids: involvement of palindromes at the recombinational loci. *Proc. Natl. Acad. Sci. U.S.A.* **72**:1373-1377.
  28. Lovett, M. A., and D. R. Helinski. 1976. Method for the isolation of the replication region of a bacterial replicon: construction of a mini-F'*km* plasmid. *J. Bacteriol.* **127**:982-987.
  29. Manis, J. J., and B. C. Kline. 1977. Restriction endonuclease mapping and mutagenesis of the F sex factor replication region. *Mol. Gen. Genet.* **152**:175-182.
  30. McHattie, L. A., and J. A. Shapiro. 1978. Chromosomal integration of phage  $\lambda$  by means of a DNA insertion element. *Proc. Natl. Acad. Sci. U.S.A.* **75**:1490-1494.
  31. Meyer, R., G. Boch, and J. Shapiro. 1979. Transposition of DNA inserted into deletions of the Tn5 kanamycin resistance element. *Mol. Gen. Genet.* **171**:7-13.
  32. Meyers, J. A., D. Sanchez, L. P. Elwell, and S. Falkow. 1976. A simple agarose gel electrophoretic method for the identification and characterization of plasmid deoxyribonucleic acid. *J. Bacteriol.* **127**:1529-1537.
  33. Novick, R. P., R. C. Clowes, S. N. Cohen, R. Curtiss III, N. Datta, and S. Falkow. 1976. Uniform nomenclature for bacterial plasmids: a proposal. *Bacteriol. Rev.* **40**:168-189.
  34. Shapiro, J. A. 1979. Molecular model for the transposition and replication of bacteriophage Mu and other transposable elements. *Proc. Natl. Acad. Sci. U.S.A.* **76**:1933-1937.
  35. Skurray, R. A., M. S. Guyer, K. Timmis, F. Cabello, S. N. Cohen, N. Davidson, and A. J. Clark. 1976. Replication region fragments cloned from *Flac*<sup>+</sup> are identical to *EcoRI* fragment f5 of F. *J. Bacteriol.* **127**:1571-1575.
  36. Skurray, R. A., N. Willetts, and P. Reeves. 1976. Effect of *tra* mutations on F-factor-specified immunity to lethal zygosis. *Mol. Gen. Genet.* **146**:161-165.
  37. Templin, A., S. R. Kushner, and A. J. Clark. 1972. Genetic analysis of mutations indirectly suppressing *recB* and *recC* mutations. *Genetics* **72**:205-215.
  38. Templin, A., L. Margossian, and A. J. Clark. 1978. Suppressibility of *recA*, *recB*, and *recC* mutations by nonsense suppressors. *J. Bacteriol.* **134**:590-596.
  39. Toussaint, A., and M. Faelen. 1973. Connecting two unrelated DNA sequences with a Mu dimer. *Nature (London) New Biol.* **242**:1-4.
  40. Warren, G., and D. Sherratt. 1977. Complementation of transfer deficient ColE1 mutants. *Mol. Gen. Genet.* **151**:197-201.
  41. Watanabe, T. 1963. Infective heredity of multiple drug resistance in bacteria. *Bacteriol. Rev.* **27**:87-115.
  42. Willetts, N. S. 1972. Location of the origin of transfer of the sex factor F. *J. Bacteriol.* **112**:773-778.
  43. Willetts, N. S., A. J. Clark, and B. Low. 1969. Genetic location of certain mutations conferring recombination deficiency in *Escherichia coli*. *J. Bacteriol.* **97**:244-249.
  44. Williams, R. C. 1977. Use of polylysine for adsorption of nucleic acids and enzymes to electron microscope specimen films. *Proc. Natl. Acad. Sci. U.S.A.* **74**:2311-2315.