JOURNAL OF BACTERIOLOGY, Nov. 1984, p. 642–650
0021-9193/84/110642-09$02.00/0

Vol. 160, No. 2

Spontaneous Deletion of Citrate-Utilizing Ability Promoted by Insertion Sequences

NAOTAKA ISHIGURO* AND GIHEI SATO

Department of Veterinary Public Health, Obihiro University of Agriculture and Veterinary Medicine, Obihiro, Hokkaido 080, Japan

Received 14 November 1983/Accepted 27 July 1984

The citrate utilization (Cit^+) transposon Tn*3411* was shown to be flanked by directly repeated sequences ($IS3411_L$ and $IS3411_R$) by restriction enzyme analysis and electron microscope observation. Cit^- deletion mutants were frequently found to be generated in pBR322::Tn*3411* by intramolecular recombination between the two copies of IS*3411*. The flanking IS*3411* elements of Tn*3411* were shown to be functional insertion sequences by Tn*3411*-mediated direct and inverse transposition. Tn*3411*-mediated inverse transposition from pBR322::Tn*3411* to the F-plasmid derivative pED100 occurred more efficiently than that of direct transposition of the Cit^+ determinant. This was thought to be due to the differential transposability of $IS3411_L$ and $IS3411_R$ in the transposition process. The frequency of transposition of IS*3411* marked with a chloramphenicol resistance determinant was much higher than IS*3411*-mediated cointegrate formation, suggesting that replicon fusions are not essential intermediates in the transposition process of Tn*3411* or IS*3411*. Spontaneous deletions occurred with high frequency in *recA* hosts. The spontaneous deletion promoted by homologous recombination between two IS*3411* elements in Tn*3411* was examined with deletion mutants.

The importance of transposons in the widespread distribution of drug-resistant bacteria has been well recognized (4, 6, 26). Also some biochemical determinants such as fermentation of lactose (8), mercury resistance (25), and production of heat-stable toxin (24) have been shown to be encoded by transposons. Most transposons are classified into three groups on the basis of mechanistic properties, genetic organization, and DNA sequence homologies (17). One group (class I) consists of the composite transposons that are flanked by insertion sequence (IS) elements such as IS*1*, IS*50* and IS*10*; the second group (class II) is related to Tn*3*; the third group (class III) is composed of the transposing bacteriophages such Mu and D108. Transposons and insertion sequences also promote a number of genetic rearrangements such as deletions, duplicative inversions, and replicon fusions (cointegrate formation). These DNA rearrangements do not require RecA function in the host cell.

Previously, we have examined over 20 citrate utilization (Cit) plasmids in Cit^+ *Escherichia coli* strains isolated from clinical and environmental sources to determine the transposability of Cit^+ gene to the other replicons. A citrate utilization transposon, Tn*3411*, was identified on a Cit plasmid, pOH3001, isolated from a strain of human origin (15). This transposon had a characteristic structure which contained two pairs of inverted repeats in the inverse orientation in the internal region of Tn*3411* (15). The other characteristic of pBR322::Tn*3411* was the formation of small deletions in *recA* strains. The formation of deletions from pOH2 seems to be due to the structure of Tn*3411*, which involes two pairs of inverted repeats, but little is known about the reason for their high rate of formation in *recA* strains. The citrate utilization transposon Tn*3411* was shown to contain two directly repeated copies of an insertion sequence, IS*3411*, and is therefore a class I composite element.

We have studied in detail the genetic and physical properties of the spontaneous deletion mutant plasmids derived from pOH2. In the present report, we show that the deletions are generated from pOH2 by intramolecular recombination between IS elements located in the direct orientation on Tn*3411*. An explanation of the deletion mechanism in Tn*3411* is also considered.

MATERIALS AND METHODS

Bacterial strains, bacteriophage, and plasmids. *E. coli* K-12 derivatives and plasmids used in this study are listed in Tables 1 and 2, respectively. Strain 433 is a *del* mutant of 483 which was kindly provided from Nevers and Saedler (20). The male-specific phage f2 was employed as an indicator phage to detect the presence of the F-derivative plasmid pED100 (same as pOX38) (29). Plasmid pOH11 is a derivative of pBR322, which specifies resistance to ampicillin and kanamycin, constructed by the insertion of *Hin*dIII-*Sal*I fragment of Tn*5* (21) between the *Hin*dIII and *Sal*I sites on pBR322 (Table 2). The plasmid pED100, which was constructed in vitro by circularization of the largest *Hin*dIII fragment of the plasmid F, was kindly provided by Willetts and Johnson (29). It carries the transfer operon and the genes responsible for autonomous replication, but is missing all known insertion sequences carried by F except for a small region of IS*3*.

Media and antibiotics. Penassay broth (Difco Laboratories) was usually used for routine bacterial growth. Cultures for phage preparation were grown in L broth (18). L agar was used for drug resistance determinations. The selective medium used for citrate utilization was Simmons citrate agar (Eiken) supplemented with appropriate nutritional requirements (15). The concentrations of antibiotics used were as follows: ampicillin, 50 μg/ml; chloramphenicol, 25 μg/ml; kanamycin, 25 μg/ml; rifampin, 50 μg/ml; streptomycin, 12.5 μg/ml for plasmid determinants and 500 μg/ml for selecting chromosomal determinants of JC1557 or JC1569 in the mating experiments; sulfonamide, 800 μg/ml; tetracycline, 12.5 μg/ml.

* Corresponding author.

TABLE 1. *E. coli* strains

Strain	Genotype[a]	Reference
JC1557	*leu his arg met lac mal gal man rpsL*	
JC1569	*recA1* mutant of strain JC1557	15
SG8	Rif^r mutant of strain C600 *thi thr leu lac*	
SG11	*recA* mutant of strain SG8	
483	Δ(*proAB-lac*) *rpsL nalA galTN116*::IS*1*	20
433	*del* mutant of strain 483	20

[a] Genotype symbols are the same as those used by Bachmann et al. (2).

Conjugation and transformation. Plasmids were introduced into *E. coli* strains by conjugation or by transformation (15).

Determination of the frequency of deletions and stability tests. Single colonies of *E. coli* strains carrying the plasmids to be tested were inoculated into Penassay broth without antibiotics and grown overnight at 37°C. The bacterial cultures were diluted by a factor of 10^6 and incubated overnight at 37°C. This serial subculturing was performed five times. Cultures were diluted in saline and plated on nonselective medium. Over 100 colonies were tested for plasmid-mediated characters. Each culture was lysed by a rapid method (14); cleared lysates were prepared and electrophoresed to test for the presence of small deletion plasmids.

Mating procedure for the measurement of transposition and cointegrate formation. Plasmid conjugation experiments were performed as described previously (15). The mating system for determination of transposition or cointegrate formation was carried out with pBR322::Tn*3411* or pOH11::Tn*3411* derivatives as transposon donors and Sa or pED100 conjugal plasmids as recipient replicons (11). Samples of 0.5 ml of donor strain SG11 and 5 ml of recipient culture of JC1569 were mixed and incubated either for 3 h or overnight at 37°C. The frequencies of transposition or cointegrate formation were estimated from duplicate experiments. The frequency of transfer of pED100 was measured by screening 50 Sm^r colonies of JC1569 for sensitivity to the male-specific phage f2 or by detecting the plasmid band of pED100 by electrophoresis. The frequency of transposition was expressed as the frequency of transfer of Cit$^+$, Ap^r, Cm^r, Km^r, or Tc^r compared with the frequency of the transfer of plasmid Sa or pED100.

Preparation of phage and plasmid DNAs. Phage DNA was extracted as described by Miller (19). The rapid and simple preparation of plasmid DNA from 5 ml of bacterial culture was carried out by the method of Holmes and Quigley (14). The lysates were extracted with phenol and treated with RNase and then cleaved with restriction enzymes or electrophoresed directly. Large preparative isolation and purification of plasmid DNA has been described (15).

Restriction enzyme analysis and electrophoresis. Restriction endonucleases (*Acc*I, *Bam*HI, *Bgl*II, *Hin*dIII, *Hin*cII, *Pst*I, *Kpn*I, *Pvu*II, *Sal*I, *Sma*I) were purchased from Takara Shuzo (Kyoto) and used according to the manufacturer's recommendations.

Agarose gels (0.8 to ~1.5%) in electrophoresis buffer (0.089 M Tris–0.0025 M disodium EDTA–0.089 M boric acid, pH 8.3) were run in the horizontal apparatus (15). Standard fragments in kilobases for gel electrophoresis were *Hin*dIII-cleaved λ phage DNA and five fragments of pBR322 (1,595, 1,361, 756, 375, and 275 base pairs [bp]) formed by cleavage with the enzymes *Acc*I, *Bam*HI, *Eco*RI, and *Pst*I.

Extraction of plasmid DNA from agarose gel and ligations. Extraction of plasmid DNA bands or restriction enzyme-generated DNA fragments from agarose gel was performed by electroelution in dialysis bags. Ligation was carried out in 10 ml of dithiothreitol–7 mM $MgCl_2$–1 mM ATP–10 mM Tris (pH 7.8). Three units of T4 DNA ligase (Takara Shuzo) was

TABLE 2. Bacterial plasmids

Plasmid	Phenotype[a]	Reference
pBR322	Ap^r Tc^r	27
pSC101	Tc^r	
pED100	Self-ligated *Hin*dIII fragment of F, tra$^+$ f2 phage sensitive	29
Sa	tra$^+$ Cm^r Km^r Sm^r Su^r	28
pOH2	pBR322::Tn*3411* Cit$^+$ Ap^r Tc^r	15
pOH3	pBR322::Tn*3411* Cit$^+$ Ap^r Tc^r	15
pOH7	Self-cloning of pOH2 with *Bam*HI, Cit$^+$ Ap^r	15
pOH9	pBR322::IS*3411*, spontaneous deletion in pOH2, Ap^r Tc^r	This paper
pOH10	pBR322::IS*3411*, spontaneous deletion in pOH3, Ap^r Tc^r	This paper
pOH11	Insertion of a *Hin*dIII-*Sal*I fragment from Tn*5* conferring Km^r into pBR322, Ap^r Km^r	This paper
pOH12	Sa::Tn*3411* tra$^+$ Cit$^+$ Cm^r Km^r Sm^r Su^r	This paper
pOH13	pOH11::Tn*3411* Cit$^+$ Km^r	This paper
pOH14	pOH11::IS*3411*, spontaneous deletion in pOH13, Km^r	This paper
pOH17	Insertion of a *Bgl*II fragment from Tn*10* conferring Tc^r into pOH13, Km^r Tc^r	This paper
pOH18	pOH11::IS*3411*, spontaneous deletion in pOH17, Km^r	This paper
pOH19	Self-reconstituted plasmid of pOH17 with *Bgl*II, Tc^r Km^w	This paper
pOH22	*Hin*dIII-A and -B ligated plasmid of pOH13, Cit$^+$ Km^r	This paper
pOH23	Spontaneous deletion in pOH22, Km^r	This paper
pOH24	Insertion of a *Hin*dIII fragment from Sa conferring Cm^r into pOH9, Ap^r Cm^r Tc^r	This paper
pOH33	Self-reconstituted plasmid of pOH17 with *Eco*RI, Km^r	This paper
pOH34	*Eco*RI-A fragment ligated plasmid of pOH17, Km^r	This paper
pOH35	*Sal*I-A and -B ligated plasmid of pOH13, Cit$^+$	This paper
pOH36	Spontaneous deletion in pOH35	This paper
pOH37	Deletion plasmid of *Pvu*II-C fragment from pOH2, Cit$^+$ Ap^r Tc^r	This paper
pOH38	Spontaneous deletion in pOH37, Ap^r Tc^r	This paper
pP2.11	pBR322 carrying Tn*9* cloned in the *Pvu*II site together with 132 bp of plasmid F	This paper

[a] Phenotype symbols: Cit$^+$, citrate utilization; Ap^r, ampicillin resistance; Cm^r, chloramphenicol resistance; Km^r, kanamycin resistance; Sm^r, streptomycin resistance; Su^r, sulfonamide resistance; Tc^r, tetracycline resistance; tra$^+$, conjugative transferability; Km^w, resistant to kanamycin at low concentration (50 μg/ml) (21).

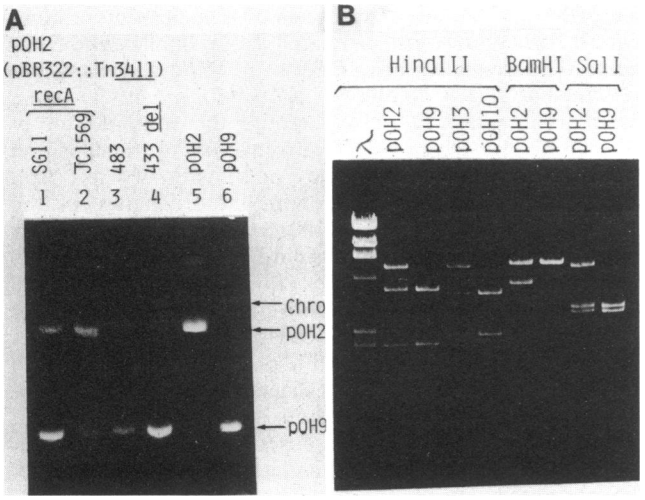


FIG. 1. Isolation of spontaneous deletion. (A) Agarose gel electrophoresis of deletion spontaneously derived from pOH2 in various strains. Host strains: *recA* strain of SG11; 2, JC1569 3, 483; 4, 433. CsCl-ethidium bromide purified plasmids in host strain JC1569: 5, pOH2; 6, deletion plasmid pOH9 from pOH2. Chro, Chromosomal band. (B) Restriction digests of deletion and pBR322::Tn*3411*. pOH9 was spontaneous deletion mutant from pOH2 and pOH10 was from pOH3. Molecular weight standards were *Hin*dIII-cleaved phage λ DNA.

added, and the mixture was incubated overnight at 15°C and used to transform $CaCl_2$-treated *E. coli* cells as described previously (15).

Electron microscopy. Plasmid DNA used for electron microscope heteroduplex analysis was cleaved by *Eco*RI. The method used to form heteroduplex molecules was essentially as described previously (15). Determination of the length of single- and double-stranded regions of the heteroduplex molecules was made by comparison with single- and double-stranded pBR322 molecules (4,362 bp) (27).

RESULTS

Isolation and characterization of deletions. In a previous paper (15), we described a small plasmid band that appeared when DNA of plasmid pBR322::Tn*3411* pOH2 was applied for gel electrophoresis. This small plasmid was also found in *Eco*RI-cleaved pOH2 and pOH3. Its molecular weight was larger than that of pBR322, indicating that small plasmid has not resulted from the precise excision of Tn*3411*. To determine whether this small plasmid is, indeed, generated from pOH2 or pOH3, pOH2 and pOH3 plasmid DNA was purified by extraction from agarose gels and used for transformation to the *recA* strains SG11 and JC1569. Although all transformants selected for Cit^+ were both Ap^r and Tc^r, 1 out of 50 Ap^r colonies selected was Tc^r, but did not utilize citrate. The plasmid content of the Ap^r Tc^r colony was analyzed by agarose gel electrophoresis and appeared indistinguishable from the small plasmid from pOH2. This plasmid was designated as pOH9 (Fig. 1A, lane 6). The remaining 49 Cit^+ Ap^r Tc^r colonies contained a mixture of DNAs of pOH2 and the deletion mutant (Fig. 1A, lanes 1 and 2).

The frequencies of spontaneous deletion from pOH2 are dependent to the growth conditions. The Cit^+ phenotype of *E. coli* SG11 carrying pOH2 was lost in over 50% of colonies tested during five subculturings. However, the Cit^+ ability of pSC101::Tn*3411*, which was constructed by transposition of Tn*3411* from λ *bb*::Tn*3411* (15) to pSC101, was relatively stable during five serial subculturings; only four clones containing the small deletion plasmid alone were obtained. No significant reduction in the frequency of deletion formation occurred in the *del* mutant strain 433, compared with that of strain 483 (Fig. 1A, lanes 3 and 4), suggesting that the process of deletion formation in Tn*3411* was different from that of IS*1*-mediated deletion (20).

Physical analysis of deletion mutant. Deletion mutants pOH9 and pOH10, derived from pOH2 and pOH3, respectively, were examined by restriction enzymes and heteroduplex analysis. As shown in Fig. 1B, comparison of the restriction enzyme *Hin*dIII-cleaved fragments of pOH2, pOH9, pOH3, and pOH10 revealed that *Hin*dIII fragment A disappeared in the deletion plasmids pOH9 and pOH10,

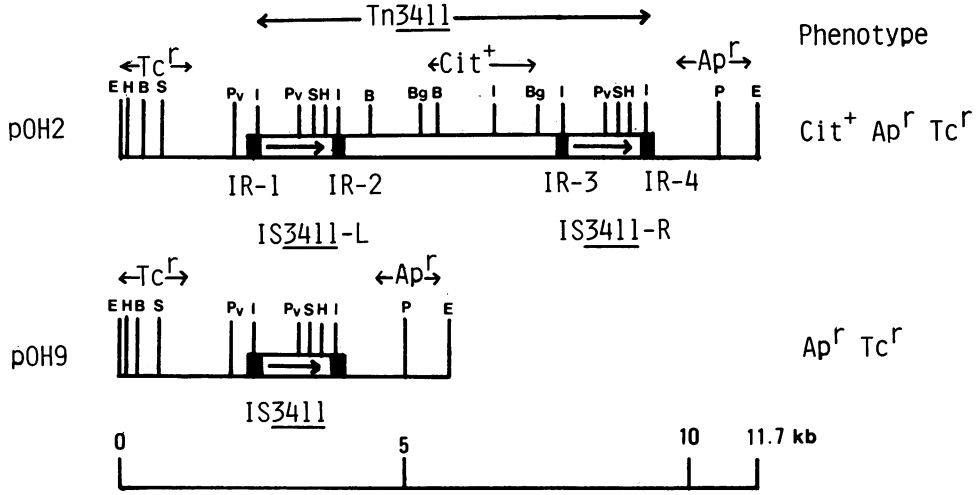


FIG. 2. Restriction maps of pOH2 and pOH9. The small closed boxes in the open boxes indicate the inverted repeats of insertion sequence IS*3411*. The inverted repeats designated as IR-1, IR-2, IR-3, and IR-4 are the same as described previously (15). The arrows indicate the orientation of IS*3411*. The distances between cleaved sites are shown in kilobases. Vertical lines indicate the cleavage sites of the following endonucleases: E, *Eco*RI; H, *Hin*dIII; B, *Bam*HI; Bg, *Bgl*II; S, *Sal*I; I, *Sma*I; P, *Pst*I; Pv, *Pvu*II. Phenotype symbols are described in Table 2, footnote *a*.

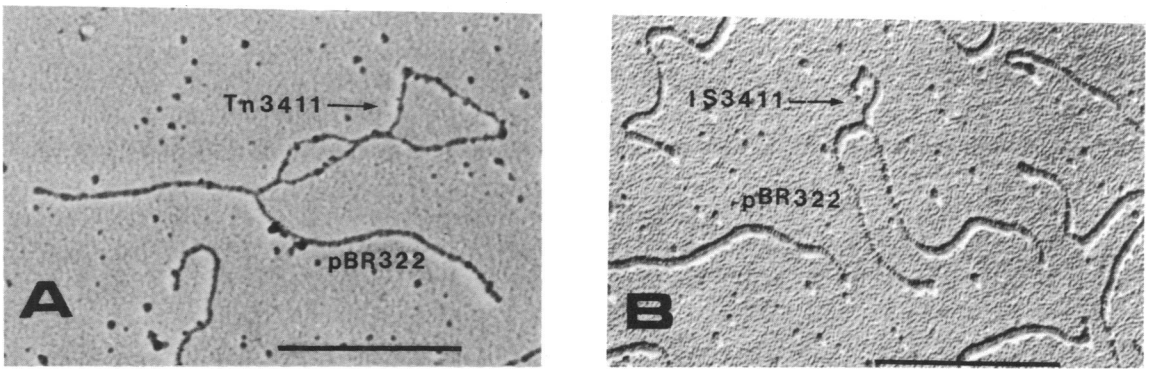


FIG. 3. DNA-DNA heteroduplexes between *Eco*RI-cleaved pOH2 and pBR322 (A) and *Eco*RI-cleaved pOH9 and pBR322 (B). The arrows indicate the loops of Tn*3411* (A) and IS*3411* (B). Bars, 0.5 μm.

whereas *Hin*dIII-B and -C remained unchanged. Analysis of pOH2 and pOH9 with *Sal*I also showed that the largest *Sal*I-A fragment disappeared in the mutant pOH9 (Fig. 1B). Plasmid pOH9 was found to have lost two fragments when digested with *Bam*HI (Fig. 1B). We have already identified the *Bam*HI, *Hin*dIII, and *Sal*I sites in pOH2 and pOH3 (15). Considering the restriction map of Tn*3411* (15), these results indicate the Cit$^-$ mutants are generated from pBR322::Tn*3411* by deletion of DNA internal of Tn*3411*.

To determine the deleted regions and junction sites on Tn*3411*, pOH2 and pOH9 were digested with single and double digests of *Eco*RI, *Bam*HI, *Pst*I, *Bgl*II, *Pvu*II, *Hin*dIII, *Sal*I, and *Sma*I, and restriction maps were constructed (Fig. 2). pOH9 was 5,700 bp in length, and the deletion did not extend into the vector plasmid pBR322. Thus, the recognition sites required for deletion formation seem to be in DNA sequences contained within Tn*3411* rather than pBR322.

One characteristic feature observed in comparison of the restriction maps of pOH2 and pOH9 is that the relative order of the *Sma*I, *Pvu*II, *Sal*I, *Hin*dIII, and *Sma*I sites in these two plasmids was the same (Fig. 2). The location of the duplicated recognition sites (indicated by arrows in Fig. 2) in restriction map of pOH2 correlated with the location of two pairs of inverted repeats in Tn*3411* identified in the heteroduplex analysis (15). Therefore, the restriction analysis of Tn*3411* indicated the presence of two 1,300-bp directly repeated sequences.

Heteroduplex analysis of deletion plasmids. As reported previously (15), heteroduplex molecules formed between pBR322 and pOH2 gave a double stem-loop structure with external inverted repeats (previously designated IR-1 and IR-4) and additional internal inverted repeats (IR-2 and IR-3) (Fig. 2, Fig. 3A). On the other hand, the heteroduplex formed between pBR322 and the Cit$^-$ deletion mutant pOH9 revealed a single stem-loop structure (Fig. 3B). pOH9 would be probably generated from pOH2 by intramolecular recombination between the two directly repeated sequences. In this recombination process, one copy of the flanking sequence remains. The size of the insert in pOH9 (1,300 bp) corresponds to that of one of the putative repeated sequences.

Demonstration of insertion sequence in Tn*3411*. To determine whether the two directly repeated sequences of Tn*3411* are functional IS elements, we measured direct and inverse transposition (5) of Tn*3411* from pOH2 to the recipient replicon Sa in mating experiments. We found that direct transposition of Cit$^+$ occurred at a frequency of 2×10^{-5} per Sa element transferred, whereas inverse transposition of Tc^r occurred at a frequency of 1×10^{-4}. To further characterize direct and inverse transposition events and also cointegrate formation promoted by the IS elements, pOH2 and pOH9 DNAs were transformed to strain SG11 harbouring pED100, and mating transposition assays were carried out. The direct or inverse transposition frequencies are expressed as the ratio of Sm^r Cit$^+$ [JC1569(pED100::Tn*3411*)] or Sm^r Ap^r Tc^r [JC1569(pED100::pBR322)] to Sm^r f2^s [JC1569(pED100)] colonies. Schematic structures of donor replicons used in the cross are shown in Fig. 4A. The values we obtained are presented in Table 3. Although no transconjugants were detected in the 3-h mating, in overnight mating direct and inverse transposition occurred at frequencies of 2×10^{-7} and 6×10^{-5}, respectively. Here the frequency of direct transposition is about 100-fold lower than that of inverse transposition (Table 3). Transconjugant plasmid DNA was characterized by agarose gel electrophoresis (data not shown). The enzyme of choice for restriction analysis of the translocated clones or cointegrates was *Hin*dIII, since this enzyme cuts pED100 once, and IS-like elements and pBR322 each have one site.

When the Cit$^-$ deletion plasmid pOH9 was used as a donor, stable cointegrates between pOH9 and pED100 were obtained of frequency of 3×10^{-6} (Table 3). From the physical and genetic analysis of pOH2 and pOH9, it was demonstrated that the citrate transposon Tn*3411* was flanked by direct duplications of putative insertion sequences (designated as IS*3411*$_L$ and IS*3411*$_R$ in Fig. 2), and that at least one copy of IS*3411* on pOH9 was a functional insertion sequence element.

Transposition and cointegrate formation promoted by Tn*3411* or IS*3411*. pOH13 was constructed by the transposition of Tn*3411* from Sa::Tn*3411*(pOH12) to pOH11. The phenotype of the resulting plasmid was Cit$^+$ Km^r because *bla* gene in pOH11 was inactivated by insertion of the transposon (Fig. 4B). The inverse transposition from pOH13 (2×10^{-5}) was estimated to be 100-fold more efficient than direct transposition (2×10^{-7}) in the 3-h mating (Table 3). In the overnight mating, the difference in frequency of direct and inverse transposition was even still marked. Of 24 Cit$^+$ transconjugants, 14 were Km^r, indicating that they carried cointegrates of pOH13 and pED100. On the other hand, all Km^r transconjugants selected were Cit$^-$, indicating that these clones were not cointegrates, but inverse transposition clones. The position of the insertion was determined by the comparison of *Hin*dIII, *Eco*RI, and *Bgl*II restriction digests of pED100, pED100::Tn*3411* and cointegrates (data not

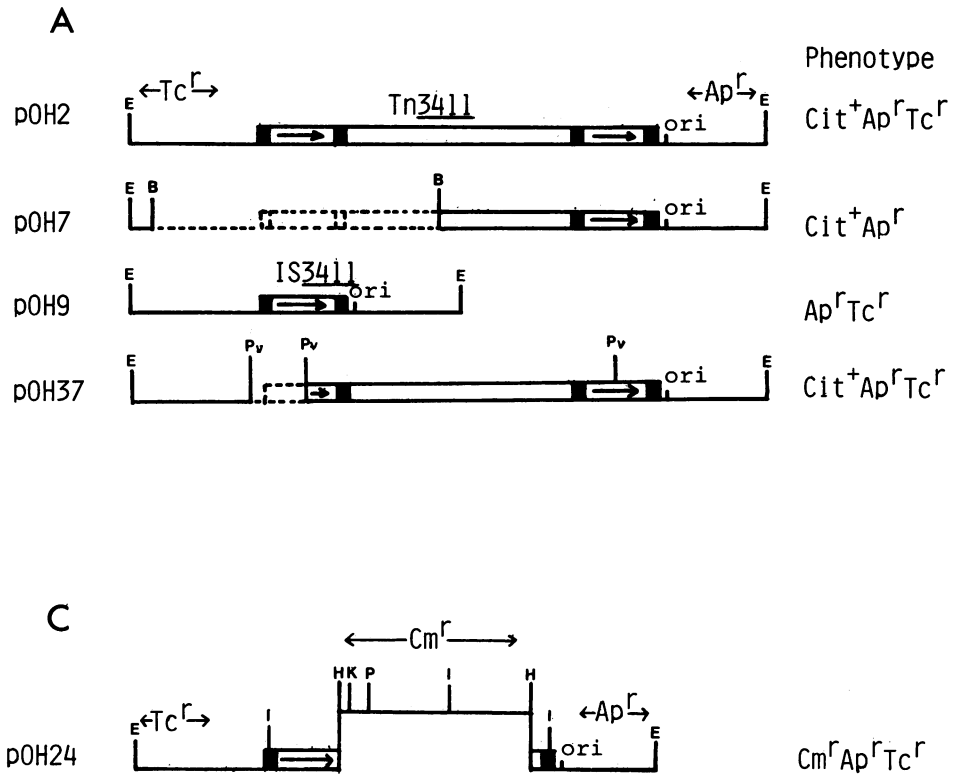


FIG. 4. Schematic representation of pOH2 and deletion derivatives of pOH2 (A), several derivative plasmids of pOH13 (B), and Cm^r-marked IS*3411* on pOH9 (C). Solid lines indicate fragments of pBR322 and the insertion fragments of Tn*5*, Tn*10*, and Sa. The unique fragments of Tn*3411* located in pBR322 are shown as the open boxes, in which the closed boxes indicate the inverted repeats of IS*3411*. Dotted lines and boxes indicate the deleted regions in the derivatives of pOH2. The arrows within the open boxes show the orientation of IS*3411*. Vertical lines indicate the cleavage sites of the following endonucleases: B, *Bam*HI; Bg, *Bgl*II; E, *Eco*RI; H, *Hin*dIII; I, *Sma*I; K, *Kpn*I; P, *Pst*I; Pv, *Pvu*II; S, *Sal*I. ori, Replication origin of pBR322.

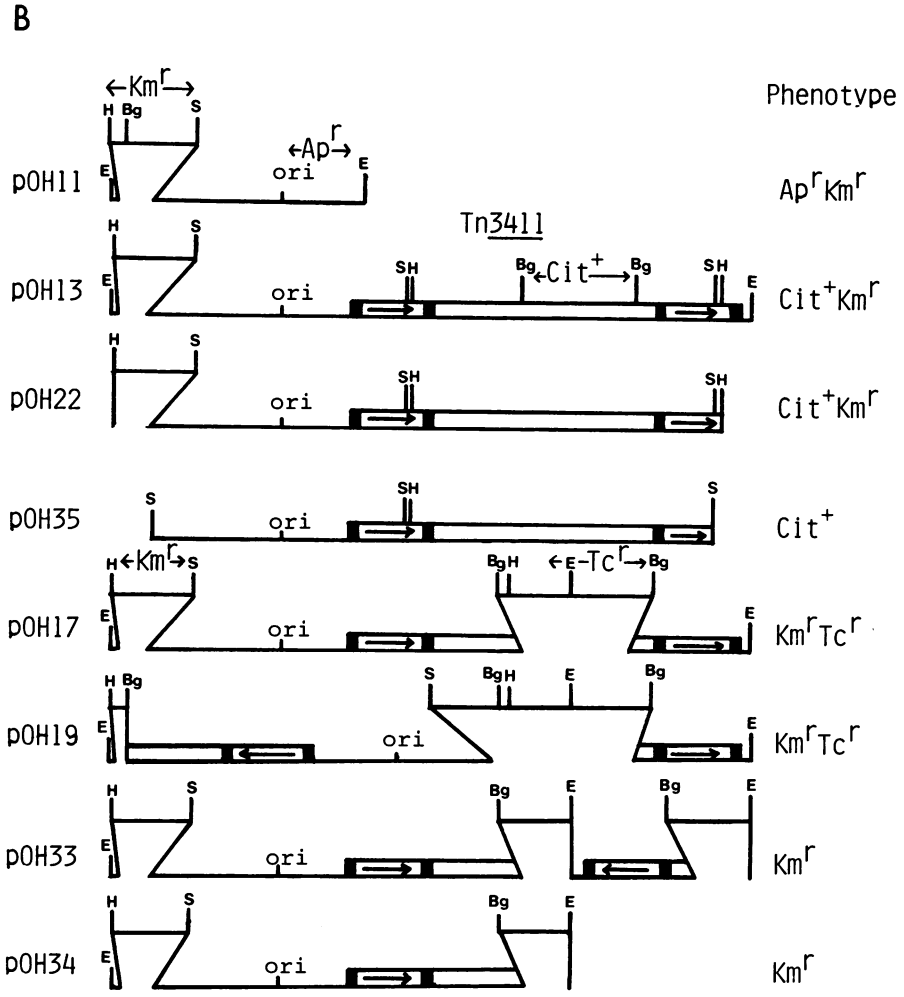
shown) (11, 29). Double selection (i.e., for Cit$^+$ and Km^r) of cointegrates was not performed.

To eliminate any difference in selection for Cit$^+$ and drug resistance, a variant of pOH13 was constructed by substructuring the *tet* gene of Tn*10* (9) for the *Bgl*II fragment coding for the Cit$^+$ determinant on Tn*3411* (Fig. 4B). Table 3 shows that the frequency of direct transposition (tetracycline selection) is at least 100-fold lower than that of inverse transposition (kanamycin selection). This result is in accord with the results of transposition assays with pOH13. Willetts and Johnson (29) have shown that when pBR322 is cloned into the plasmid pED100, not only is the copy number of the composite plasmid intermediate between the two parental plasmids, but also the transfer efficiency of the composite is approximately sevenfold higher than that of the F derivative alone. We could therefore overestimate the contribution of derivatives that carry a pBR322 origin of replication (i.e., cointegrates have received pBR322 by inverse transposition). Although we did not correct for the increased mating efficiency of the composite plasmids, we have done the transposition assay with pP2.11 as a control (11). The frequency of direct transposition monitoring the Cm^r marker of Tn*9* was higher than that of inverse transposition selecting for Tc^r, as described by Galas and Chandler (11). This result differed from the findings with pBR322 derivatives carrying Tn*3411*.

We constructed the deletion plasmids pOH22 and pOH37, which were missing the smallest *Hin*dIII and *Pvu*II fragment of pOH13 and pOH2, respectively (Fig. 4A and B). When pOH22 was used as a donor replicon, no Cit$^+$ transconjugants were obtained (Table 3), whereas the frequency of inverse transposition (kanamycin selection) was equal to that of pOH13. In the case of pOH37, no direct transposition occurred, since a part of IS3411$_L$ including the terminal region was cut with *Pvu*II enzyme and deleted from pOH2. However, Cit$^+$ Ap^r Tc^r transconjugants selected for cointegration were obtained, and they all contained three kinds of plasmid, pED100, pOH37, and a small deletion plasmid (designated as pOH38; see below) (Fig. 5).

To confirm whether high frequency of inverse transposition rather than direct transposition is due to the orientation of IS*3411* elements, pOH17 was cleaved with *Bgl*II or *Eco*RI, and recombinant plasmids (pOH19 and pOH33) carrying IS elements in the inverted orientation were constructed in vitro (Fig. 4B). The frequency of transposition of Tc^r or Km^r from pOH19 to pED100 was low (Table 3). In the case of pOH33, the frequency of direct transposition was measured at 4×10^{-6} in the overnight mating. The plasmid structures in the Km^r clones obtained were divided into two groups (direct transposition and replicon fusion).

Difference of transposition ability of IS*3411*$_L$ and IS*3411*$_R$. When pOH7 carrying IS*3411*$_R$ was used as a donor replicon in the analysis of cointegrate formation, the Cit$^+$ Ap^r colonies were obtained at the frequency of 2×10^{-8}, but all clones contained an unmodified pOH7 and a larger derivative of pED100. No stable cointegrate between pOH7 and pED100 was obtained. This mobilization seems to occur via a IS element on pOH7, because pBR322 cannot be mobilized



by the conjugation system of F (frequency of $<10^{-10}$) (11). In contrast to pOH7, when pOH34 carrying IS*3411*$_L$ was used as a donor (Fig. 4B), stable cointegrates of pOH34 and pED100 were obtained at the frequency of 10^{-5} in the overnight mating (Table 3). This result shows that the transposition functions encoded by IS*3411*$_L$ differ from those of IS*3411*$_R$.

Stability of cointegrates. Before analyzing the stability of cointegrates, the pOH13 derivatives pOH17, pOH19, pOH22, and pOH33 (Fig. 4B) were examined for stability by monitoring the loss of plasmid-mediated characters and by testing for deletion mutants in *recA* strains. Spontaneous deletions were detected in overnight cultures of pOH13, pOH17, and pOH22 (Fig. 5). On the other hand, we did not find deletions in pOH19 or pOH33 (which have inverted copies of IS*3411*). These results suggest that the appearence of deletions correlates with the orientation of IS*3411* elements located on the plasmids.

The stability of cointegrates generated by inverse transposition or replicon fusion was tested. The products of inverse transposition from pOH13 or pOH17 to pED100 yielded small plasmids (pOH14 or pOH18) with the size of pOH11::IS*3411* at high rates. This result shows that cointegrates of pED100 and pBR322 directly flanked by IS*3411*$_L$ and IS*3411*$_R$ are remarkably unstable in a *recA*$^-$ background. The other cointegrates (i.e., replicon fusions of pOH9 with pED100 and pOH34 with pED100) were tested for their stability. In the replicon fusions of pOH9 and pED100, the analysis of plasmid profiles by agarose gel electrophoresis indicated that the small plasmid was absent. A similar result was obtained in the case of pOH34. As compared with the breakdown of the cointegrates obtained in the inverse transposition, replicon fusions promoted by IS*3411* or IS*3411*$_L$ were quite stable in the *recA*$^-$ background.

Confirmation that IS*3411* is a transposable element. To further characterize the transposition mechanism of IS*3411*, the insertion sequence located in pOH9 was marked with the Cm^r determinant by insertion of the *Hin*dIII-cleaved fragment from Sa (28) into the *Hin*dIII site in IS*3411* (pOH24 in Fig. 4C), the transposability of IS*3411* Cm^r was assayed. Cm^r marker was translocated from pOH24 to pED100 at a frequency of 1×10^{-6} in the 3-h mating, whereas Ap^r Tc^r Cm^r cointegrates were obtained at a frequency of 3×10^{-8} (Table 3). The 92 transconjugants tested were resistant to chloramphenicol alone, but not resistant to ampicillin or tetracycline. This result suggests that transposition of IS*3411* marked with Cm^r occurs more efficiently than cointegrate formation promoted by IS*3411*.

Model for deletion formation by Tn*3411*. The model for deletion formation is shown in Fig. 5. Since deletions were still produced from pOH22 in which a part of the terminal

repeated sequences of IS*3411*$_R$ was deleted, all of the sequences of IS*3411* were not essential for intramolecular deletion in Tn*3411*. pOH35, which was constructed with *Sal*I (Fig. 4B), also yielded deletion (pOH36).

The other internal deletion plasmid, pOH37, was stable during serial subculture and was shown to be free of small deletion plasmids in agarose gel electrophoresis. On the other hand, when the transposition assay from pOH37 to pED100 was carried out, the isolated Cit$^+$ Ap^r Tc^r transconjugants were shown to contain a small deletion plasmid (pOH38) by gel electrophoresis (Fig. 5C). pOH38 had the *Pvu*II and *Sma*I recognition sites located in IS*3411* (data not shown). The potential recombination process that occurred during the mating experiments is shown in Fig. 5C. Probably, the cointegrates between pOH37 and pED100, which were flanked by IS*3411*$_R$, were formed during the mating process. Afterwards, these cointegrates broke down into two independent replicons. The alternative recombination between a part of IS*3411*$_L$ and IS*3411*$_R$ would proceed during the mating (Fig. 5C). The final transconjugants would contain three kinds of plasmids (i,e., pED100::IS*3411*$_R$, pOH37, and pOH38) (Fig. 5C). A similar explanation for mobilization of pOH7 with pED100 in the mating should be considered (Table 3).

DISCUSSION

In this study, our analysis shows that the Cit$^+$ determinant located on Tn*3411* is flanked by directly repeated sequences designated IS*3411*$_L$ and IS*3411*$_R$, and that Cit$^-$ deletion mutants were generated by intramolecular recombination between the direct repeats in *recA* genetic background. The evidence for this is as follows. (i) Spontaneous deletions (5.7 kilobases) that carried the recognition sites for *Sma*I, *Pvu*II, *Sal*I, and *Hin*dIII on IS*3411* were frequently isolated from pBR322::Tn*3411* in a *recA* host. (ii) The stem-loop structure in the heteroduplex molecules formed between pBR322 and deletion mutant plasmids pOH9 corresponded to the length of a single copy of IS*3411*. (iii) Tn*3411*-mediated inverse transposition of pBR322::Tn*3411* to pED100 occurred in mating experiments, and also replicon fusions between pBR322::IS*3411* and pED100 were observed. (v) Transposition of the Cm^r-marked IS*3411* element to pED100 occurred. Taken together with previous genetic and physical results (15), transposon Tn*3411* is a typical class I composite element in which the Cit$^+$ determinant is flanked by terminal directly repeated copies of IS*3411*. The terminal repeated sequences of IS*3411*$_L$ were composed of inverted repeats, which were previously designated as IR-1 and IR-2. Repeats IR-3 and IR-4 were the terminal inverted repeats for IS*3411*-R (Fig. 2).

Many insertion sequences have been identified in the composite transposon, e.g., IS*10*, IS*50*, and IS*903* occur in Tn*10*, Tn*5*, and Tn*903*, respectively. The transposability of the repeated sequences of class I transposons has been investigated by testing for inverse transposition in Tn*5* (3) and in Tn*10* (5). In this study, the frequency of IS*3411*-mediated inverse transpositon from pOH13 to pED100 occurred more efficiently than that of direct transposition of the Cit$^+$ determinant (Table 3). The transposition assay system used in this study is that described by Galas and Chandler (11), and therefore our data can be compared with the results reported in their paper. We did not correct the increased mating efficiency of pED100 cointegrates. The frequency of IS*3411*-mediated inverse transposition was 2×10^{-2} in overnight matings (Table 3). This extremely high frequency seems to be due either to the copy number of the composite plasmids which have received pBR322 origin of replication by inverse transposition or to the prolonged mating times. If the efficiency of transposition for class I transposons was dependent on the recognition sites for insertion sequence-encoded transposase in insertion elements (22), the internal inverted repeats (IR-2 and IR-3) in Tn*3411* might be more active sites in this process. However, since the insertion sequence elements IS*3411*$_L$ and IS*3411*$_R$ are situated in the direct orientation in Tn*3411*, either insertion sequence may be the active element. Moreover, the high frequency of inverse transposition might reflect different functions of IS*3411*$_L$ and IS*3411*$_R$. Indeed, IS*3411*$_L$ was shown to be a functional insertion sequence element in the cointegrate formation assay, and the resulting cointegrates were quite stable in *recA* background (Table 3). However, pOH7 carrying IS*3411*$_R$ failed to form the cointegrates with pED100. These results indicate that the two IS*3411* elements are not identical; IS*3411*$_L$ encodes the function required to promote transposition, and IS*3411*$_R$ has lost this ability. However, it seems unlikely that IS*3411*$_R$ is completely defective in transposition functions, because mobilization of pBR322 occurred via the IS*3411*$_R$ element on

TABLE 3. Frequencies of transposition and cointegrate formation between each donor replicon and recipient replicon pED100

Expt	Donor replicon	Phenotype[a] of donor	Frequencies of transposition and cointegrate formation at[b]:					
			3-h mating			Overnight mating		
			Direct[c]	Inverse[d]	Cointegrate	Direct	Inverse	Cointegrate
A	pOH2	Cit$^+$ Ap^r Tc^r	$<10^{-8}$	$<10^{-8}$	NT[e]	2×10^{-7}	6×10^{-5}	NT
	pOH7	Cit$^+$ Ap^r	NT	NT	$<10^{-8}$	NT	NT	$<10^{-8}$
	pOH9	Ap^r Tc^r	NT	NT	$<10^{-8}$	NT	NT	3×10^{-6}
	pOH37	Cit$^+$ Ap^r Tc^r	$<10^{-8}$	$<10^{-8}$	$<10^{-8}$	$<10^{-8}$	7×10^{-7}	8×10^{-8}
B	pOH13	Cit$^+$ Km^r	2×10^{-7}	2×10^{-5}	NT	1×10^{-6}	2×10^{-2}	NT
	pOH17	Km^r Tc^r	4×10^{-8}	2×10^{-5}	NT	8×10^{-8}	1×10^{-5}	NT
	pOH19	Km^w Tc^r	5×10^{-8}	NT	NT	1×10^{-6}	NT	NT
	pOH33	Km^r	$<10^{-8}$	NT	NT	4×10^{-6}	NT	NT
	pOH34	Km^r	NT	NT	$<10^{-8}$	NT	NT	1×10^{-5}
	pOH22	Cit$^+$ Km^r	$<10^{-8}$	2×10^{-5}	NT	$<10^{-8}$	2×10^{-2}	2×10^{-5}
C	pOH24	Cm^r Ap^r Tc^r	1×10^{-6}	NT	3×10^{-8}	1×10^{-5}	NT	4×10^{-7}

[a] Abbreviations for plasmid-mediated characters are as in Table 2.
[b] The frequencies are the means of independent two experiments.
[c] Direct transposition.
[d] Inverse transposition.
[e] NT, Not tested.

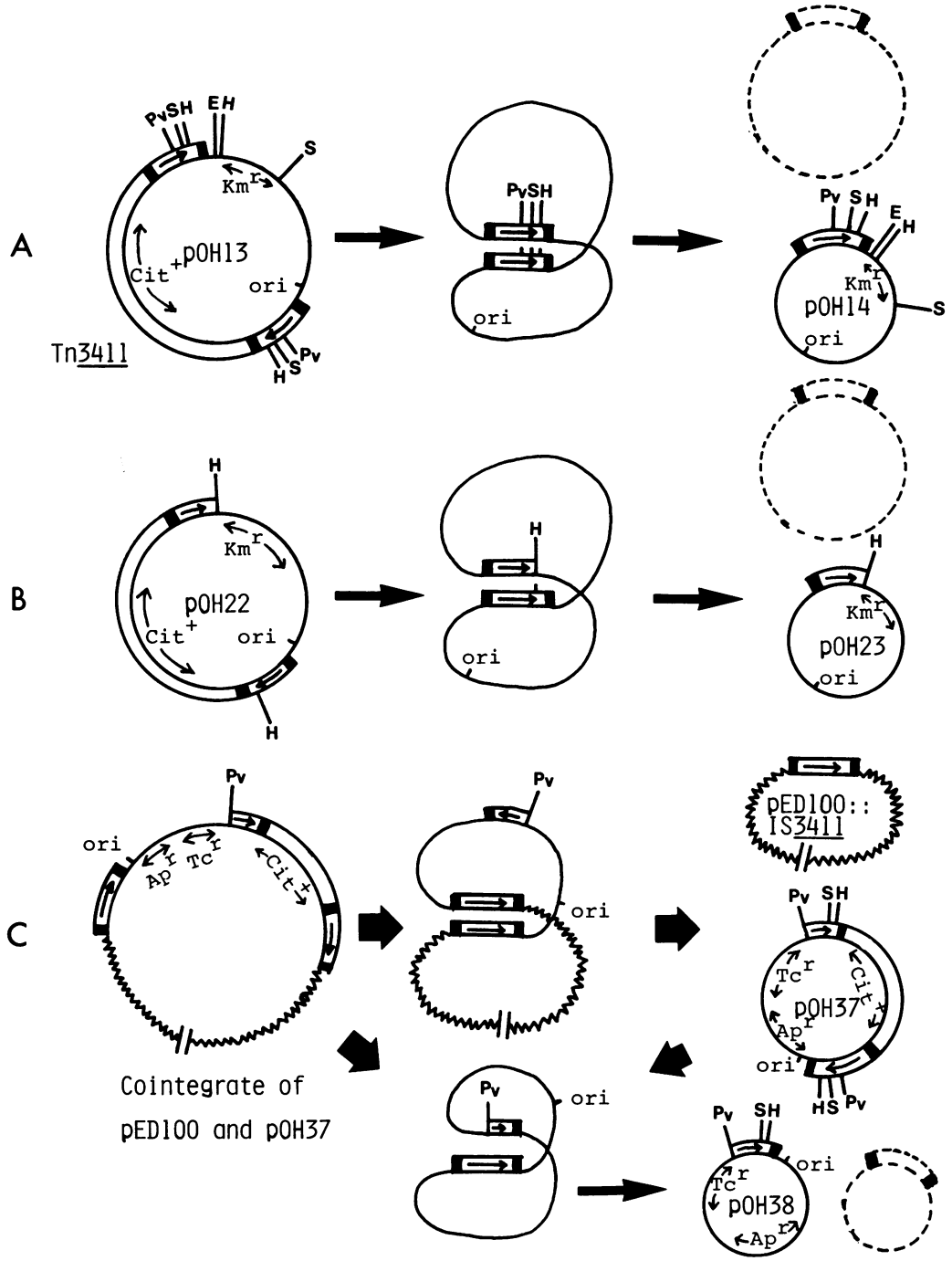


FIG. 5. Diagram of spontaneous deletion pathways. (A and B) Recombination between the two IS*3411* insertion sequences contained in pOH13 and pOH22 is diagrammed. Recombination will result in the excision of the circular DNAs (dotted lines). They will be lost because they cannot replicate. (C) Formation of deletion from pOH37 by mobilization with pED100 is diagrammed. The zigzag lines indicate the region of pED100. The open boxes indicate the region of Tn*3411*, and the closed small boxes within the open boxes indicate the inverted repeats of IS*3411*. The relative orientation of IS*3411* is given by the arrows in the open boxes. ori, Origin of pBR322. Phenotype symbols are described in Table 2, footnote *a*. Abbreviations of restriction enzymes: E, *Eco*RI; H, *Hin*dIII; Pv, *Pvu*II; S, *Sal*I.

pOH7 or on pOH37 with plasmid pED100. Detailed analysis of IS*3411*$_R$-mediated mobilization is now in progress.

Several transposition models accounting for the joining of transposon ends to a target site and for replication of the transposing element have been proposed (10, 13, 23). It could be interesting to know the steps in Tn*3411*-mediated transposition. We have made some comparisons of the transposition frequency and the frequency of cointegrate formation in several transposition assays. The frequency of Tn*3411*-mediated inverse transposition is more efficient than that of replicon fusion (Table 3) and the cointegrates due to replicon fusion form small portion of the cointegrates obtained. In addition, the frequency of transposition of Cm^r-marked IS*3411* is much higher than IS*3411*-mediated cointe-

grate formation. Taken together with the remarkable stability of IS*3411*- or IS*3411*$_L$-mediated cointegrates, this strongly suggests that replicon fusions are not essential intermediates in the transposition process of IS*3411* or Tn*3411*. Determination of the duplicated target DNA sequences generated by Tn*3411* or IS*3411* will be useful in clarifying the transposition system of these transposable elements.

The formation of Cit$^-$ deletions on pBR322::Tn*3411* remains unsolved. It appears that the spontaneous Cit$^-$ deletions are derived from pBR322::Tn*3411* by intramolecular recombination between the two direct copies of IS*3411* in Tn*3411*. However, it is obscure whether this deletion is due to an internal site-specific recombination system located on Tn*3411* or due to the homological recombination in direct repeats of IS*3411*. Two intact IS*3411* repeated sequences are not essential for the formation of deletions, because spontaneous deletion occurs in Cit$^+$ plasmids pOH22 and pOH35 in which the outer sequences were made in vitro (Fig. 5). However, the deletion plasmid, pOH37 did not yield deletions. This fact might suggest that intramolecular recombination occurs in to the left of the *Pvu*II site of IS*3411* elements. On the other hand, the deletions were isolated in the transconjugants after mating with pOH37(pED100) (Fig. 5). Considering these results, the internal site-specific recombination sites may be situated between the *Pvu*II and *Sal*I sites in IS*3411* (Fig. 2), and the polypepties required for genetic recombination would be encoded within lefthand DNA regions from *Pvu*II site in IS*3411* elements. Also the two flanking IS*3411* element are not identical in their transposition properties. The functional difference between IS*3411*$_L$ and IS*3411*$_R$ must be reflected in differences at the nucleotide sequence level.

Recently, it was reported that spontaneous deletions are easily isolated from pBR322 carrying cloned foreign DNA fragments (12, 16). The other mechanism of deletion formation in Tn*3411* is that deletion occurs at short repeated sequences in both *recA*$^+$ and *recA*$^-$ strain backgrounds (1, 7). Since the frequency of spontaneous deletion plasmids from pBR322::Tn*3411* is extremely high, it is difficult to explain the deletion mechanism in Tn*3411*. It is not yet clear whether the formation of spontaneous deletions in pBR322::Tn*3411* is enhanced by high-copy-number effects of vector plasmid pBR322. To define the deletion mechanism of Cit$^+$ determinant in Tn*3411*, determination of the nucleotide sequence of IS*3411* is needed.

ACKNOWLEDGMENTS

This work was supported in part by grants 57480077, 57770322, and 58760211 from the Scientific Research Fund of the Ministry of Education, Science and Culture of Japan.

LITERATURE CITED

1. **Albertini, A. M., M. Hofer, M. P. Calos, and J. H. Miller.** 1982. On the formation of spontaneous deletions: the importance of short sequence homologies in the generation of large deletions. Cell **29**:319–328.
2. **Bachmann, B. J., K. B. Low, and A. L. Taylor.** 1976. Recalibrated linkage map of *Escherichia coli* K-12. Bacteriol. Rev. **40**:116–167.
3. **Berg, D. E., L. Johnsrud, L. Mcdivitt, R. Ramabhadran, and B. J. Hirschel.** 1982. Inverted repeats of Tn*5* are transposable elements Proc. Natl. Acad. Sci. U.S.A. **79**:2632–2635.
4. **Calos, M. P., and J. H. Miller.** 1980. Transposable elements. Cell **20**:579–595.
5. **Chandler, M., E. Roulet, L. Silver, E. Boy de la Tour, and L. Caro.** 1979. Tn*10* mediated integration of the plasmid R100.1 into the bacterial chromosome: Inverse transposition. Mol. Gen. Genet. **173**:23–30.
6. **Cohen, S. N.** 1976. Transposable genetic elements and plasmid evolution. Nature (London) **263**:731–738.
7. **Collins, J., G. Volckaert, and P. Nevers.** 1982. Precise and nearly-precise excision of the symmetrical inverted repeats of Tn*5*; common features of *recA*-independent deletion events in *Escherichia coli*. Gene **19**:139–146.
8. **Cornelis, G., D. Ghosal, and H. Saedler.** 1978. Tn*951*: a new transposon carrying a lactose operon. Mol. Gen. Genet. **160**:215–224.
9. **Foster, T. J., M. A. Davis, D. E. Roberts, K. Takeshita, and N. Kleckner.** 1981. Genetic organization of transposon Tn*10*. Cell **23**:201–213.
10. **Galas, D. J., and M. Chandler.** 1981. On the molecular mechanisms of transposition. Proc. Natl. Acad. Sci. U.S.A. **78**:4858–4862.
11. **Galas, D. J., and M. Chandler.** 1982. Structure and stability of Tn*9*-mediated cointegrates: evidence for two pathways of transposition. J. Mol. Biol. **154**:245–272.
12. **Garaev, M. M., A. F. Bobkov, A. F. Bobkova, V. N. Kalinin, V. D. Smirnov, Yu. K. Khudakov, and T. I. Tikchonenko.** 1982. The site-specific deletion in plasmid pBR322. Gene **18**:21–28.
13. **Harshey, R. M., and A. I. Bukhari.** 1981. A mechanism of DNA transposition. Proc. Natl. Acad. Sci. U.S.A. **78**:1090–1094.
14. **Holmes, D. S., and M. Quigley.** 1981. A rapid boiling method for the preparation of bacterial plasmids. Anal. Biochem. **114**:193–197.
15. **Ishiguro, N., G. Sato, C. Sasakawa, H. Danbara, and M. Yoshikawa.** 1982. Identification of citrate utilization transposon Tn*3411* from a naturally occurring citrate utilization plasmid. J. Bacteriol. **149**:961–968.
16. **Jones, I., S. B. Primrose, and S. D. Ehrlich.** 1982. Recombination between short direct repeats in a *recA* host. Mol. Gen. Genet. **188**:486–489.
17. **Kleckner, N.** 1981. Transposable elements in prokaryotes. Annu. Rev. Genet. **15**:341–404.
18. **Lennox, E. S.** 1950. Transduction of linked genetic characters of the host by bacteriophage P1. Virology **1**:190–206.
19. **Miller, J.** 1973. Experiments in molecular genetics. Cold spring Harbor Laboratory, Cold Spring Harbor, N.Y.
20. **Nevers, P., and H. Saedler.** 1978. Mapping and characterization of an *E. coli* mutant defective in IS*1*-mediated deletion formation. Mol. Gen. Genet. **160**:209–214.
21. **Rothstein, S. J., and W. S. Reznikoff.** 1981. The functional differences in the iverted repeats of Tn*5* are caused by a single base pair nonhomology. Cell. **23**:191–199.
22. **Sasakawa, C., and D. E. Berg.** 1982. IS*50*-mediated inverse transposition: discrimination between the two ends of an IS element. J. Mol. Biol. **159**:257–271.
23. **Shapiro, J. A.** 1979. Molecular model for the transposition and replication of bacteriophage Mu and other transposable elements. **76**:1933–1937.
24. **So, M., F. Heffron, and B. J. McCarthy.** 1979. The *E. coli* gene encoding heat stable toxin is a bacterial transposon flanked by inverted repeats of IS*1*. Nature (London) **277**:453–456.
25. **Stanisch, V. A., P. M. Bennett, and M. H. Richmond.** 1977. Characterization of a translocation unit encoding resistance to mercuric irons that occurs on a nonconjugative plasmid in *Pseudomonas aeroginosa*. J. Bacteriol. **129**:1227–1233.
26. **Starlinger, P.** 1980. IS elements and transposons. Plasmid **3**:241–259.
27. **Sutcliff, J. G.** 1979. Complete nucleotide sequence of the *Escherichia coli* plasmid pBR322. Cold Spring Harbor Symp. Quant. Biol. **43**:77–90.
28. **Tait, R. C., R. C. Lundquist, and C. I. Kado.** 1982. Genetic map of the crown gall suppressive IncW plasmid pSa. Mol. Gen. Genet. **186**:10–15.
29. **Willetts, N., and D. Johnson.** 1981. pED100, a conjugative F plasmid derivative without insertion sequences. Mol. Gen. Genet. **182**:520–522.