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Occurrence and Properties of Composite Transposon Tn*2672*: Evolution of Multiple Drug Resistance Transposons

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We found Tn*2671* (the 23-kb long IS*1*-flanked r-determinant of NR1-Basel) inserted into the ampicillin resistance gene *bla* of the Tn*3*-related transposon Tn*902*. The resulting 28-kilobase-long composite transposon Tn*2672* (= Tn*902* *bla*::Tn*2671*) is stable, and it translocates as a unit into various loci including IS*1* of the resistance transfer factor of R100-1. These results are discussed with respect to the evolution of R plasmids providing multiple drug resistance.

Transposons carried by resistance plasmids often contain several drug resistance determinants. For example, the IS*1*-flanked r-determinant (r-det) of R1 determines resistance to chloramphenicol (Cm^r), fusidic acid (Fa^r), streptomycin (Sm^r), sulfonamide (Su^r), ampicillin (Ap^r), and kanamycin (Km^r) and contains the Ap^r transposon Tn*3*, the Ap^r Sm^r Su^r transposon Tn*4*, and the Km^r transposon Tn*2350* (Fig. 1; 6, 23, 24). Since terminal IS*1* elements can potentially mediate the transposition of any DNA sequence (19, 20, 28, 30) the entire r-det of R1 should also be a transposon. Similarly, the IS*1*-flanked r-det of NR1 (or R100) specifying Cm^r, Fa^r, Sm^r, Su^r, and resistance to mercury (Hg^r) is called transposon Tn*2670* and includes the Sm^r Su^r Hg^r transposon Tn*21* (Fig. 1; 14, 19a, 23, 24). By comparing the structure of these and other related R plasmids, it has been speculated that sequential acquisition of individual transposons by a plasmid results in the formation of such multiple resistance transposons (8, 24).

In studies of transposition of Tn*2671* (= r-det of NR1-Basel) which is a Tn*2670* derivative containing a 1.25-kilobase (kb) IS element called IS*30* (1, 17, 19, 19a), we have isolated a bacteriophage P7 derivative carrying Tn*2671* (19a). The site of insertion of Tn*2671* in the P7 genome was in the *bla* gene for Ap^r, i.e., within transposon Tn*902* (18, 19a, 33). Here we present evidence that Tn*902* *bla*::Tn*2671* is stable and transposes as a unit. This composite transposon is called Tn*2672* (Fig. 1). Furthermore, we have observed the transposition of Tn*2672* into other transposable DNA elements. These results provide examples for the evolution of composite transposons with multiple drug resistance determinants.

MATERIALS AND METHODS

Bacterial strains, phages, and plasmids. Table 1 lists the bacterial strains, phages, and plasmids used. The resistance transfer factor (RTF) carried by strain LC524 is a derivative of R100-1 carrying one copy of IS*1* and Tn*10* (3). Both phages P7 and P7*bla*::r-det204 carry the thermoinducible *cI ts*48 mutation (31). Plasmids pHC230, 238, 240, and 241 are Ap^r derivatives of pHC219, 220, 221, and 222, respectively.

Media. Tris-glucose minimal (TG) medium, LBMg medium, LB agar, and Davis minimal agar containing sulfathiazol were described before (17, 25). Antibiotics were added to LB agar without glucose and $CaCl_2$ at the following concentrations: ampicillin (150 μg/ml), chloramphenicol (25 μg/ml), $HgCl_2$ (25 μg/ml), nalidixic acid (80 μg/ml), rifampin (80 μg/ml), streptomycin (20 μg/ml), and tetracycline (25 μg/ml).

Isolation and characterization of RTF::Tn*2672*. Bacteria lysogenic for P7::r-det204 express P7 immunity and P1-specific restriction and modification. Since the P7*bla*::r-det204 genome is not packageable intact into P7 phage particles, defective killer particles and few plaque-forming phages (less than about 1% of the phage particles) are produced upon heat induction (19a).

LC524 cells grown in TG medium with appropriate supplement were treated with $CaCl_2$ and infected with P7*bla*::r-det204 particles at a multiplicity of about 10^{-4} plaque-forming phages per cell as described previously (17). Cm^r transductants were collected and mated with strain CH102 as described before (15, 19a), and Cm^r Rif^r transconjugants were isolated. From these, Cm^r Su^r Hg^r Tc^r colonies were screened for absence of P7 immunity, P1-specific restriction and modification, and production of killer particles (17). These Cm^r Su^r Hg^r Tc^r Rif^r transconjugants were further mated with strain CH101, and Cm^r Sm^r Su^r Hg^r Tc^r Nal^r colonies were isolated. From the resulting cells, plasmids were isolated and subjected to restriction cleavage (2) and electron microscopic analysis.

Electron microscopy. Plasmid DNA was alkali denatured and after neutralization was immediately mounted for electron microscopy by the formamide-cytochrome *c* technique of Davis et al. (10). PM2 DNA and fd DNA were added as length standards for double- and single-stranded DNA, respectively. However, the snap-back structure of Tn*10* (11, 22) within RTF provided another, intramolecular length reference.

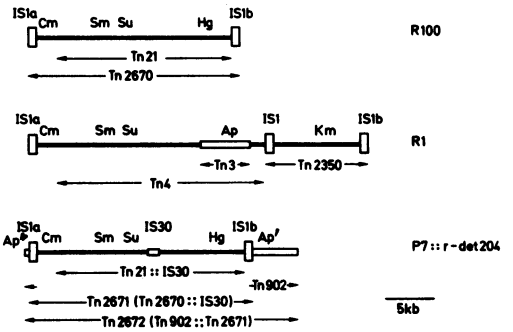


FIG. 1. Schematic representation of composite transposons found in r-det of R100, R1, and P7::r-det204. The r-det of R100 and R1 carry two and three directly repeated IS*1*s, respectively (7, 14). The IS*1*-mediated transposons Tn*2350*, Tn*2670*, and Tn*2671* were described by Clerget et al. (6) and Iida et al. (19a). Tn*2671* is the r-det of NR1-Basel containing IS*30* (1, 19a). Tn*3*, Tn*4*, and Tn*21* in the r-det were described by Kopecko and co-workers (23, 24). Tn*902* is a relative to Tn*3* and was found in phage P7 (18, 33). Tn*902 bla*::Tn*2671* found in P7::r-det204 was described by Iida et al. (19a). Ap′ and Ap″ in Tn*2672* indicate the split parts of the Ap^r gene.

Electron micrographs were taken with a Philips EM 301 microscope at 40 kV and a magnification of 10,000. DNA molecules were measured from 10×-enlarged negatives by using a Numonics digitizer.

RESULTS

Transposition of Tn*902 bla*::Tn*2671* from P7 to RTF. Recently, we described the integration of Tn*2671* into the *bla* gene of Tn*902* carried in the phage P7 genome (Fig. 1; 19a). Here we present evidence that the composite structure Tn*902 bla*::Tn*2671* is able to translocate from the P7 derivative P7::r-det204 to plasmid RTF.

Since Tn*902 bla*::Tn*2671* is Ap^s, we first used the markers carried by Tn*2671* to detect the transposition of Tn*902 bla*::Tn*2671*. Because P7::r-det204 is an oversized specialized transducing phage for Cm^r, Sm^r, Su^r, and Hg^r, the majority of Cm^r transductants obtained by infection with P7::r-det204 at low multiplicity of infection was expected to carry the transposed r-det segment (17; 19a). Cm^r transductants appeared with a frequency of about 2 per plaque-forming phage when the LC524 recipient cells carrying RTF were infected with P7::r-det204 at a multiplicity of 10^{-4} PFU per cell. To distinguish a Cm^r marker on the RTF plasmid from one transposed to the host chromosome or still in the P7 prophage, Cm^r transductants were collected and mated with the Rif^r strain CH102. About half of the Cm^r Rif^r transconjugants showed no P7 immunity, no P1-specific restriction and modification, no production of killer particles upon induction and had a Cm^r Su^r Hg^r Tc^r phenotype. Furthermore, these drug resist-

TABLE 1. Bacterial strains, phages, and plasmids[a]

Designation	Relevant characters		Reference or source
	Genotype	Phenotype	
Escherichia coli K-12			
LC524	*leu thy* Δ(*lac-proB*) (RTF)		L. Caro
CH101	*thr met leu hsd*$_K$ *nal*		Derived from WA921 (32)
CH102	*rpo str* P1^r		Derived from W3110
Phages			
P7*c1ts48*	Tn*902*	Ap^r	(31)
P7*bla*::r-det204*c1ts48*	Tn902*bla*::Tn*2671* = Tn*2672*	Ap^s Cm^r Sm^r Su^r Hg^r	(19a)
Plasmids			
pHC219	RTF::Tn*2672*	Tc^r Ap^s Cm^r Sm^r Su^r Hg^r	This study
pHC220	RTF::Tn*2672*	Tc^r Ap^s Cm^r Sm^r Su^r Hg^r	This study
pHC221	RTF::Tn*2672* (IS*1*::Tn*2672*)	Tc^r Ap^s Cm^r Sm^r Su^r Hg^r	This study
pHC222	RTF::Tn*2672*	Tc^r Ap^s Cm^r Sm^r Su^r Hg^r	This study
pHC230	RTF::Tn*902*	Tc^r Ap^r, derived from pHC219	This study
pHC238	RTF::Tn*902*	Tc^r Ap^r, derived from pHC220	This study
pHC240	RTF::Tn*902*	Tc^r Ap^r, derived from pHC221	This study
pHC241	RTF::Tn*902*	Tc^r Ap^r, derived from pHC222	This study
pHC257	RTF*tet*Δ::*cat*$^+$ *aad*$^+$ *sul*$^+$ *mer*$^+$	Tc^s Ap^s Cm^r Sm^r Su^r Hg^r	This study
pHC258	RTF::Tn*2672 tet*	Tc^s Ap^s Cm^r Sm^r Su^r Hg^r	This study

[a] Phage P7 carries the Ap^r transposon Tn*902* (18, 33). P7*bla*::r-det204 is P7*bla*::Tn*2671*, which carries the Tn*2671* inserted within the *bla* gene of Tn*902*.

ance determinants could be transferred together to the Nal^r strain CH101. The resulting Cm^r Sm^r Su^r Hg^r Tc^r Nal^r transconjugants yielded Ap^r Cm^s Sm^s Su^s Hg^s Tc^r Nal^r colonies with a frequency of 2×10^{-10} to 9×10^{-10}. This could be explained by precise excision of the r-det (Tn*2671*) from the Tn*902 bla*::Tn*2671*. This characterization suggested that the Cm^r Sm^r Su^r Hg^r Tc^r Nal^r transconjugants were derivatives of strain CH101 carrying the plasmid RTF::Tn*902 bla*::Tn*2671*. This was the first evidence that Tn*902 bla*::Tn*2671* is a composite transposon, and we call it Tn*2672*.

Localization of Tn*2672* on four RTF plasmid derivatives. To physically verify the transposition of Tn*2672* as a unit from the P7 genome to RTF, four independent RTF::Tn*2672* derivatives, plasmids pHC219, 220, 221, and 222, were subjected to restriction cleavage analysis. As Fig. 2 shows, the four internal *Bam*HI fragments b, c, d and e, derived from Tn*2672* (Fig. 3C), appeared in all four plasmids. This, and in particular the presence of the *Bam*HI fragment b (junction between Tn*902* and Tn*2671*) confirmed that Tn*2672* transposed into RTF as a unit. The data in Fig. 2 indicate that the Tn*2672* in all four plasmids examined is inserted within *Bam*HI fragment A of RTF. The sites and the orientation of Tn*2672* were more precisely mapped by restriction cleavage analysis with *Bgl*II, *Eco*RI, and *Hin*dIII (data not shown). The results of this analysis were confirmed by *Pst*I cleavage of the RTF::Tn*902* plasmids pHC230, 238, 240, and 241 (Fig. 2) which had been derived from RTF::Tn*2672* by precise excision of Tn*2671*. These restriction cleavage analyses revealed that all four plasmids examined contain Tn*2672* in a relatively narrow region of RTF and that pHC221 carries the insertion in its IS*1* (Fig. 3B). Furthermore, the two IS*1* elements of the r-det (Tn*2671*) carried by pHC219 and pHC221 are opposite in orientation to that of RTF, whereas pHC220 and pHC222 carry all three IS*1* elements in the same orientation.

To independently determine the site of insertion of Tn*2672* within the IS*1* of pHC221, purified pHC221 DNA was denatured, rapidly renatured, and observed in the electron microscope for intramolecular renatured regions (snap backs). Taking the orientation of Tn*2672* relative to the resident IS*1* into account, it was expected that besides Tn*10* two other snap backs should appear; the size of the two stems should add up to the length of IS*1* (0.77 kb; 26), and the added size of the two loops should correspond to that of Tn*902* plus one IS*1* element (5.7 kb; (19a). The distance between these two structures should be equivalent to the r-det minus two IS*1*s (approximately 22 kb; 19a), and the distance of

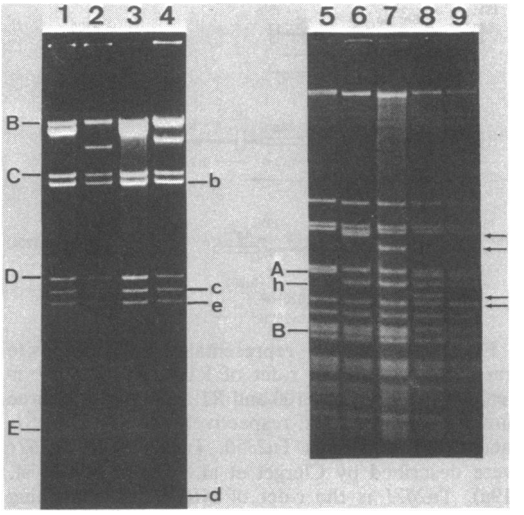


FIG. 2. Electrophoresis patterns of plasmids after treatment with restriction endonucleases. Slots 1 through 4 show fragments produced by *Bam*HI cleavage of various RTF::Tn*2672* plasmids. Electrophoresis was carried out in 0.8% agarose. Capital and small letters identify restriction cleavage fragments derived from RTF and Tn*2672*, respectively, as illustrated in Fig. 3B and C. Slots: 1, pHC220; 2, pHC219; 3, pHC222; 4, pHC221. The largest fragment A from RTF disappeared due to the Tn*2672* insertion. The two junction fragments containing part of the RTF and Tn*2672* segments were not labeled, since their size varies from one plasmid to the other. In slot 1 both junction fragments migrated roughly to the same position below B. In slot 2 one junction fragment migrated with B, and the other migrated between B and C. Slots 5 through 9 show fragments produced by *Pst*I cleavage of various RTF::Tn*902* derivatives from RTF::Tn*2672*. Electrophoresis was carried out in 1.3% agarose. Slots: 5, RTF; 6, pHC238 derived from pHC220; 7, pHC241 derived from pHC222; 8, pHC240 derived from pHC221; 9, pHC230 derived from pHC219. Capital letters A and B identify the two restriction cleavage fragments of RTF containing IS*1* sequences (Fig. 3B). Fragment h was the largest *Pst*I fragment derived from the internal part of Tn*902*. The same fragment also appeared from Tn*2672* (Fig. 3C). The arrows indicate the larger junction fragments containing part of RTF and Tn*2672* segments. The other junction fragments are either too small to be seen or overlap with another fragment.

one of the snap backs from Tn*10* should amount to 14.4 kb (14). In fact, such a structure was easily detected, and the measurements correspond to the lengths predicted (Fig. 4A and B). The insertion site of Tn*2672* within IS*1* was mapped 0.57 kb from its left-hand end. This is in good agreement with the result from the restriction cleavage analysis. Similarly, the site of insertion and orientation of Tn*2672* in pHC219

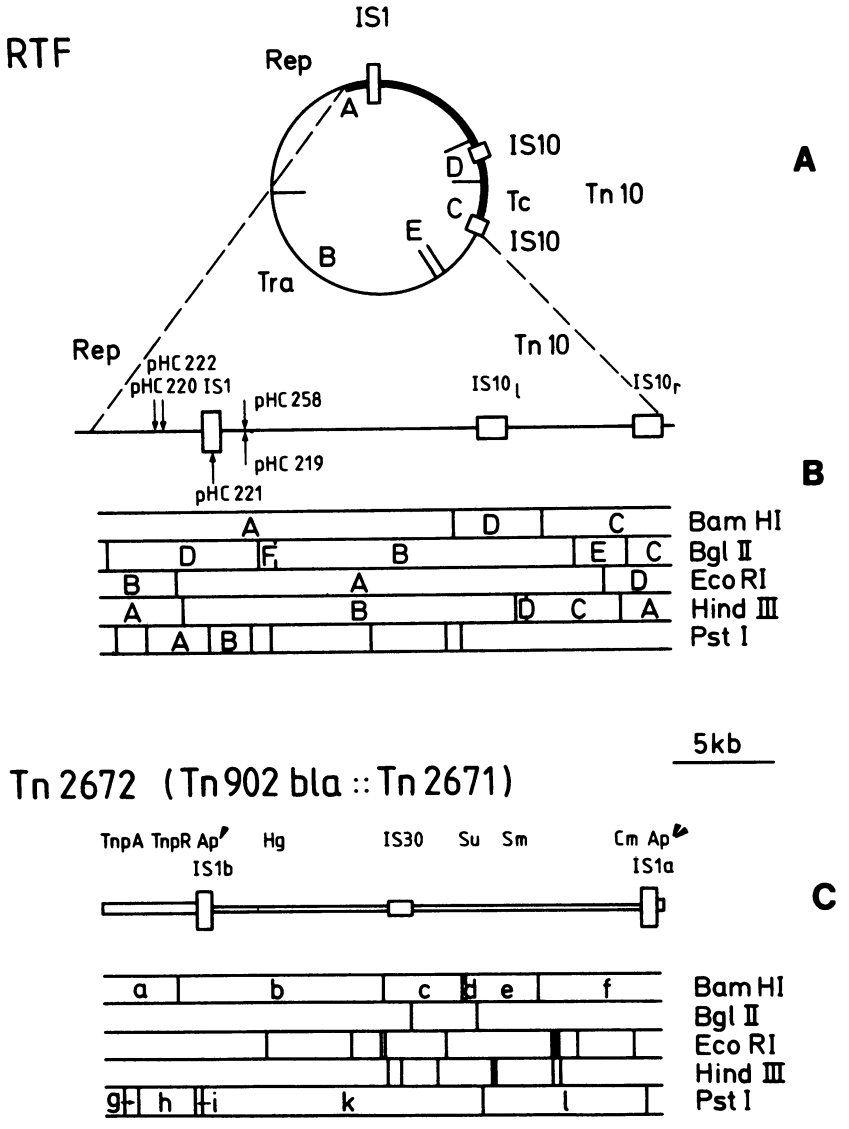


FIG. 3. Restriction cleavage maps of RTF and Tn*2672*. A, *Bam*HI map of RTF. The segment drawn in thick line is shown again in B. This diagram also identifies all cleavage sites for *Bgl*II, *Eco*RI, *Hin*dIII, and *Pst*I, as deduced from the maps of the parental plasmid R100-1 (16). Except for the *Pst*I map, the fragments were labeled in alphabetical order according to their size. IS*10*$_l$ and IS*10*$_r$ indicate, respectively, the left and right copy of IS*10* on this map. The sites of insertion of Tn*2672* in RTF derivatives are indicated by arrows. Drawing of these arrows from above indicates that all three IS*1*s of the transposition derivatives are carried in the same orientation. For plasmids carrying their Tn*2672* in the opposite orientation, the arrow is drawn from below. C, The restriction cleavage maps of Tn*2672* are based on the cleavage maps of Tn*902* and Tn*2671* (1, 18, 19a). We have also taken advantage of the published nucleotide sequence of IS*1* and of the Tn*902*-related Ap^r transposon Tn*3* (13, 26). Since the small *Pst*I fragment i containing 180 base pairs of IS*1* is about 320 base pairs long, the insertion site of Tn*2671* within the Ap^r gene *bla* must lie approximately 140 base pairs to the right of the *Pst*I site between fragments h and i. Standard symbols are used for the identification of genetic functions. TnpA, TnpR, Ap', and Ap" are located on Tn*902*, and IS*1*b, Hg^r, IS*30*, Su^r, Sm^r, Cm^r, and IS*1*a are located on Tn*2671*. The 5-kb scale applies to B and C.

was confirmed by electron microscopic analysis of snap-back structures formed by annealing of the inverted copies of IS*1* (data not shown).

Insertion of drug resistance markers into and the restructuring of Tn*10* on RTF. We have also looked for an RTF derivative carrying the transposon inserted within the Tc^r gene of Tn*10*. Ap^s Cm^r Sm^r Su^r Hg^r Tc^s transconjugants were isolated in the same way as described for Ap^s Cm^r Sm^r Su^r Hg^r Tc^r transconjugants. Among those, two independently isolated clones which carried pHC257 and pHC258 were analyzed in detail. Strain CH101(pHC258) gave Cm^r Sm^r Su^r Hg^r Tc^r revertants with a frequency of 10^{-9}, suggesting that the inactivation of the Tc^r gene was not due to the insertion of Tn*2672*. Indeed, restriction cleavage analysis and electron microscopy revealed that Tn*2672* was inserted near the IS*1* of RTF (Fig. 3) and that no significant structural alteration was observed in the Tn*10* region of pHC258. Thus, pHC258 appears to carry a spontaneous point mutation in the Tc^r gene.

Neither Tc^r nor Ap^r revertants ($<10^{-11}$) were found from strain CH101(pHC257). Restriction cleavage analysis of pHC257 indicated that deletion of part of Tn*10* had occurred after insertion of either Tn*2671* or Tn*2672* (in the latter case, the *tnpA* and *tnpR* part of Tn*902* was also deleted) into the region common to fragments *Bgl*II-E and *Eco*RI-D (Fig. 3B). The presence of a deletion was also indicated by the snap-back structure observed in single-stranded pHC257 DNA (Fig. 4C). Although the IS*10* stem appeared intact, the size of the loop measured 23.7 kb (Fig. 4D), which is less than expected for Tn*10*::Tn*2671* or Tn*10*::Tn*2672*, but which corresponds to the value expected from the restriction analysis. We therefore interpret the genotype of pHC257 to be RTF*tet*Δ::*cat*$^+$ *aad*$^+$ *sul*$^+$ *mer*$^+$ (Table 1). The deletion formation might have been mediated either by IS*1*b or by IS*10*$_l$ as defined in Fig. 3.

DISCUSSION

We have demonstrated that the composite transposon Tn*2672*, which consists of an IS*1*-flanked r-det (Tn*2671*) inserted into the *bla* gene of the Tn*3*-related transposon Tn*902*, translocates as a unit from the P7 genome to a transferable R plasmid. Tn*3* is known to contain the three genes *tnpA*, *tnpR*, and *bla* of which only *tnpA* and *tnpR* are involved in the transposition process (5, 12, 13). Since the structure of Tn*902* resembles that of Tn*3*, (18, 33), an insertion into the *bla* gene is not expected to inactivate the Tn*902* transposition functions. We therefore conclude that Tn*902* transposition functions are preserved in Tn*2672* and promote also the transposition of the composite transposon Tn*2672*. In our selection of transposition products only the Cm^r marker was involved. Therefore, not only the transposition of Tn*2672* but also that of Tn*2671* (r-det) could have been detected. However, five of six RTF derivatives examined which had acquired the Cm^r Sm^r Su^r Hg^r markers carried an intact Tn*2672*. This indicates that transposition of Tn*2672* occurs more frequently than that of Tn*2671*.

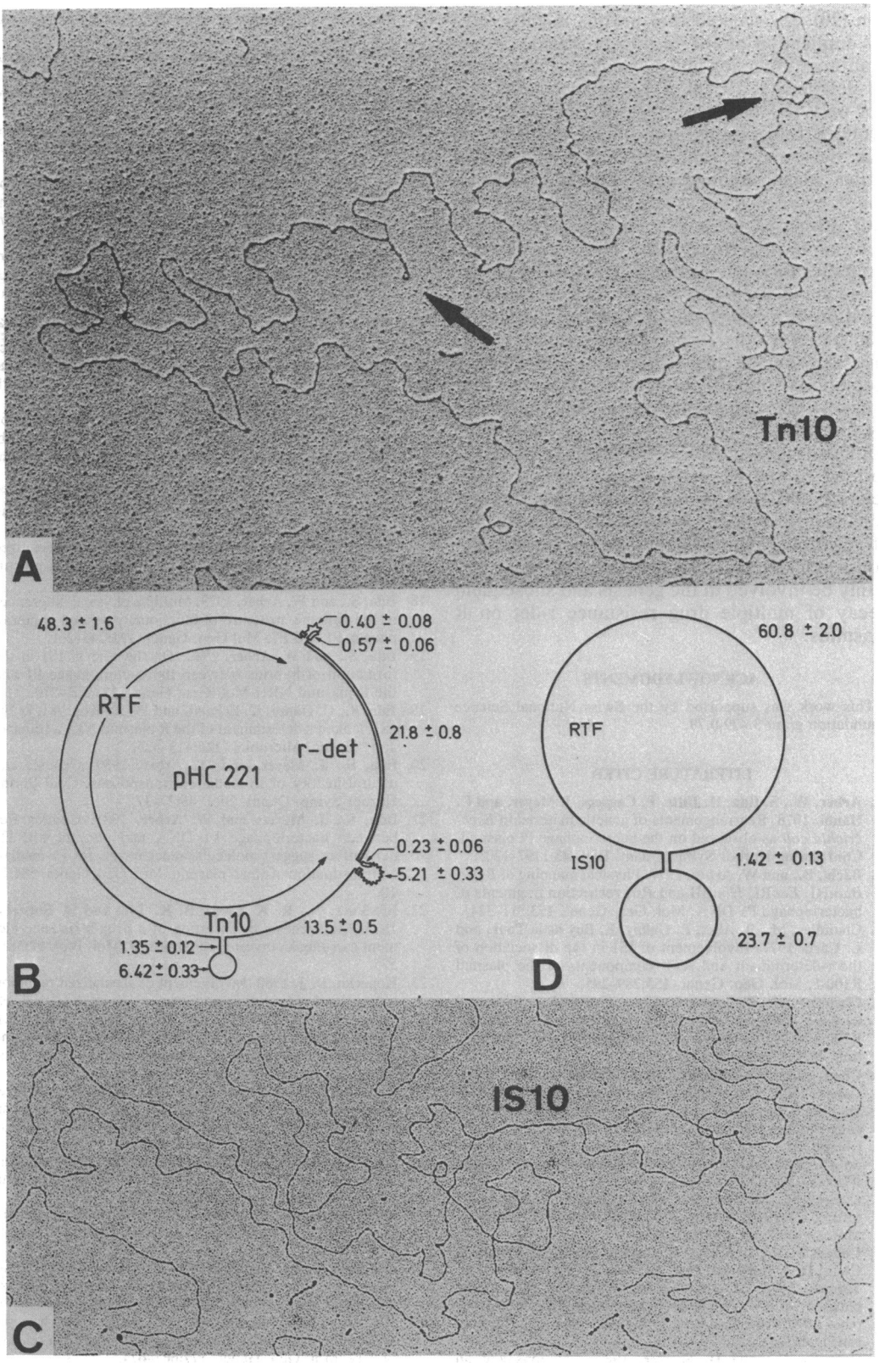
Tn*2672* does not confer Ap^r, but it contains a split *bla* gene which can be reconstituted by precise excision of Tn*2671*. Therefore, via transposition of Tn*2672* a silent *bla* gene can spread to other replicons and later become reactivated.

We observed that the composite transposon Tn*2672* (or perhaps Tn*2671*) inserted into two other transposable elements, Tn*10* and IS*1*. We do not know yet whether these composite structures are able to transpose. The transposition of Tn*10* is mediated by the flanking IS*10* (4, 11). Since the two copies of IS*10* appear to have remained intact, the Tn*10* *tet*Δ::*cat*$^+$ *aad*$^+$ *sul*$^+$ *mer*$^+$ probably still behaves as a transposon. This deletion derivative could illustrate yet another aspect of r-det evolution. Provided that Tn*2672* had transposed, the deletion of Tn*902* sequences abolishes its transposition functions and thus stabilizes the insertion within Tn*10*(Δ).

Similarly, IS*1* derivatives having inserted at the unique *Pst*I site either a Km^r fragment or pBR322 were shown to transpose as a unit (21, 29). It would be interesting to know whether the IS*1*::Tn*2672* also behaves as a transposon. Translocation of this composite structure might also be brought about by *recA*-mediated reciprocal recombination between either part of the split IS*1* and an IS*1* resident on the target replicon, by a translocation mechanism discussed by Iida et al. (19, 21).

Previously we have demonstrated that a DNA sequence could become an IS*1*-flanked transpo-

FIG. 4. Snap-back structures observd in RTF::Tn*2672* plasmids. A, Electron micrograph of a single-stranded pHC221 molecule showing three snap-back structures: one representing Tn*10* and two formed by the IS*1* elements flanking the r-det annealing to the split resident IS*1* of RTF (arrows). B, Interpretation of the structure shown in A. Length measurements (in kb) of the various regions are based on 16 molecules. Tn*902* sequences are indicated by a wavy line. C, Electron micrograph of single-stranded pHC257 DNA documenting the snap-back structure formed by IS*10* and the increase in loop size of Tn*10*. D, Length measurements (in kb) of the snap-back structure shown in C, based on 13 molecules. They are interpreted to indicate integration of Tn*2671* or Tn*2672* within the unique part of Tn*10* and deletion of a DNA segment.


Tn10
A
48.3 ± 1.6
0.40 ± 0.08
0.57 ± 0.06
RTF
r-det
21.8 ± 0.8
pHC 221
0.23 ± 0.06
5.21 ± 0.33
Tn10
13.5 ± 0.5
1.35 ± 0.12
6.42 ± 0.33
B
60.8 ± 2.0
RTF
IS10
1.42 ± 0.13
23.7 ± 0.7
D
IS10
C

son (20). IS*1*-flanked transposons might evolve by duplication of IS*1* in transpositional cointegration or inversion and subsequent IS*1*-mediated deletion (9, 19, 20, 27). We speculated that not only IS*1* but also other IS elements could produce transposons in a similar manner, and that these newly generated transposons could either transpose into another unrelated transposon or serve as a target for different transposons (20). Either of these processes can result in a new composite transposon. Here we describe examples for such processes and for the further evolution of composite transposons: stable translocation of a composite transposon, its integration into yet other transposable elements, precise excision of one component, and deletion formation within the composite transposon. Further restructuring might occur which is mediated by transposable elements within or outside the composite transposon. The stability of newly formed composite transposons depends on the relative activities of the participating transposons and IS elements as well as on the efficiency of reciprocal recombination acting on homologous sequences. These mechanisms must certainly be involved in the genesis and subsequent decay of multiple drug resistance r-det on R plasmids.

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