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Evolution of Complex Resistance Transposons from an Ancestral Mercury Transposon

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The molecular interrelationship of a transposon family which confers multiple antibiotic resistance and is assumed to have been generated from an ancestral mercury transposon was analyzed. Initially, the transposons Tn*2613* (7.2 kilobases), encoding mercury resistance, and Tn*2608* (13.5 kilobases), encoding mercury, streptomycin, and sulfonamide resistances, were isolated and their structures were analyzed. Next, the following transposons were compared with respect to their genetic and physical maps: Tn*2613* and Tn*501*, encoding mercury resistance; Tn*2608* and Tn*21*, encoding mercury, streptomycin, and sulfonamide resistance; Tn*2607* and Tn*4*, encoding streptomycin, sulfonamide, and ampicillin resistance; and Tn*2603*, endocing mercury, streptomycin, sulfonamide, and ampicillin resistance. The results suggest that the transposons encoding multiple resistance were evolved from an ancestral mercury transposon.

It is well known now that movable genetic elements such as transposons or insertion sequence elements have played a large part in the generation and evolution of bacterial plasmids. In particular, the results of numerous reports on transposons encoding resistance to various antibiotics or heavy metals strongly suggest that the sequential transposition of such segments promotes rapid evolution of R plasmids (11, 12). Genetically distinguishable resistance determinants in many cases have been shown to be composed of structurally and functionally individual transposons, e.g., Tn*3* (9), Tn*5* (18), Tn*9* (1), and Tn*10* (7). On the other hand, several multiple-resistance transposons encoding phenotypically different resistance determinants also have been identified, e.g., Tn*4* (13), Tn*7* (2), Tn*554* (15), Tn*2603* (25), Tn*21* (12), Tn*1691* (19), Tn*1699* (19), and Tn*2610* (T. Yamamoto, M. Watanabe, K. Matsumoto, and T. Saiwai, Mol. Gen. Genet., in press). The transposition of these multiple-resistance transposons must be involved in the dissemination and rapid accumulation of multiple-resistance determinants. From an evolutionary viewpoint, it seems instructive to pursue how the resistance genes could have acquired the ability to transpose. In the last few years, it has become obvious that the naturally occurring transposons fall into distinctive classes on the basis of their genetic and physical organization. One of these is a class flanked by structurally and functionally intact insertion sequence-like elements at both ends, e.g., Tn*5* (18), Tn*9* (1), and Tn*10* (7). This evidence suggests a natural history in which transposons are formed when an antibiotic resistance gene is flanked by two nearby, identical insertion sequence elements. Another transposon class is represented by Tn*3* in which the functions required for its transposition are specified in the central region, although intact terminal DNA repeat structures are necessary for transposition (9). These two transposon groups can be thought of as separate evolutionary classes of transposons (11).

A multiple-resistance transposon, Tn*2603*, which is thought to have played a major role in the distribution of the type II β-lactamases gene in nature (25), also is an element whose genes required for transposition are encoded in the central portion of the transposon (22). Tn*2603* encodes resistance to streptomycin, sulfonamide, and mercury in addition to that for ampicillin. Such transposons encoding multiple resistance would seem, from an evolutionary viewpoint, more highly evolved and more advanced than transposons conferring a single resistance determinant. On the basis of a fine structural analysis of Tn*2603* (24), it was hypothesized previously that a certain mercury resistance transposon was responsible for the evolution of a family of multiple-resistance transposons, including Tn*2603*. To confirm this hypothesis, we isolated transposons encoding mercury resistance and mercury, streptomycin, and sulfonamide resistance from two kinds of naturally occurring plasmids and compared their physical structures with various, apparently related, multiple-resistance transposons. The data in this report suggest that certain antibiotic

resistance transposons have evolved from a common ancestral mercury transposon.

MATERIALS AND METHODS

Bacterial strains and plasmids. The bacterial strains and plasmids used were *Escherichia coli* K-12 CRT46 Rif^r, a rifampin-resistant mutant of CRT46 (*dnaA thy thr leu thi lac tonA supE*), AB2463 (*recA thr leu thi lac gal xyl mtl pro his arg rpsL tsx sup*), C600 (*thr leu thi lac tonA supE*), and ML1410 (*met nal*). RGN823 is a conjugative plasmid originally isolated from *Klebsiella pneumoniae* (16), belonging to the incompatibility group P, which mediates resistance to mercury, streptomycin, sulfonamide, chloramphenicol, tetracycline, kanamycin, and ampicillin. pCS229 is a conjugative plasmid encoding resistance to mercury, streptomycin, sulfonamide, chloramphenicol, and ampicillin and was originally isolated from *Proteus mirabilis* (Yamamoto et al., in press). The plasmids, pMK1 (ColE1::Tn*5*), pUB781 (ColE1::Tn*501*), and pSC179 (pSC101::Tn*4*) were kindly provided by H. Ogawa, University of Osaka, Osaka, Japan, P. M. Bennett, University of Bristol, Bristol, England (3), and D. J. Kopecko, Walter Reed Army Medical Center, Washington, D.C. (13), respectively. The ColE1 plasmid was also used.

Genetic and physicochemical methods. Genetic transformation, conjugal plasmid transfer, plasmid DNA preparation, restriction endonuclease digestion, and DNA heteroduplex formation were conducted according to previously described methods (24, 25; Yammamoto, in press).

RESULTS AND DISCUSSION

In a previous paper, it was concluded from a physical comparison of transposons that Tn*2603* is closely related to other multiple-resistance transposons: i.e., Tn*4*, encoding resistance to ampicillin, streptomycin, and sulfonamide, and Tn*21*, encoding resistance to streptomycin, sulfonamide, and Hg (24). An evolutionary history could be hypothesized for these transposons in which an ancestral mercury-resistance transposon evolved through the acquisition of streptomycin and sulfonamide resistance determinants, followed by the acquisition of an ampicillin resistance determinant. A mercury transposon, Tn*501*, has been identified and characterized previously (3). However, its physical structure is appreciably different from that of transposons Tn*2603*, Tn*21*, or Tn*4*; i.e., it appeared to be evolutionarily unrelated. Therefore, to confirm the above hypothesis, we first tried to isolate other naturally occurring transposons determining Hg resistance alone or in combination with the streptomycin and sulfonamide resistance determinants.

Identification of a transposon, Tn*2608*, determining Hg, streptomycin, and sulfonamide resistance. To detect the potential transposability of the Hg or streptomycin resistance gene of plasmid RGN823, the plasmid was first integrated into the chromosome of *dnaA* strain CRT46 Rif^r by integrative suppression as described by Nishimura et al. (14). The resultant thermoresistant clones exhibited all phenotypes for resistance to Hg, streptomycin, sulfonamide, chloramphenicol, kanamycin, tetracycline, and ampicillin determined by RGN823 at 44°C. The integrated state of the plasmid in the thermoresistant clone was also verified by a lack of detectable autonomous DNA. Then, the CRT46 Rif^r derivative with an integrated RGN823 was transformed with ColE1. Plasmid DNA prepared from this strain was used to transform *E. coli* C600. Transformants were selected for ColE1^r Hg^r or ColE1^r Sm^r (streptomycin resistance) and were further examined for resistance to ampicillin, kanamycin, chloramphenicol, and tetracycline. Clones exhibiting Cm^r, Ap^r, Km^r, and Tc^r in addition to the other selected markers were suspected of harboring both plasmids of ColE1 and RGN823 due to the excision of autonomous RGN823 from the integrated state and were discarded. Twenty-six and 20 transformants selected for ColE1^r Hg^r and ColE1^r Sm^r, respectively, exhibited simultaneous resistance to Hg, streptomycin, and sulfonamide. Plasmid DNAs prepared from six clones of the Hg^r Sm^r Su$^+$ (sulfonamide resistance) ColE1^r type were digested with *Eco*RI endonuclease and electrophoresed on a 0.8% agarose gel (Fig. 1).

All DNAs had three common fragments of

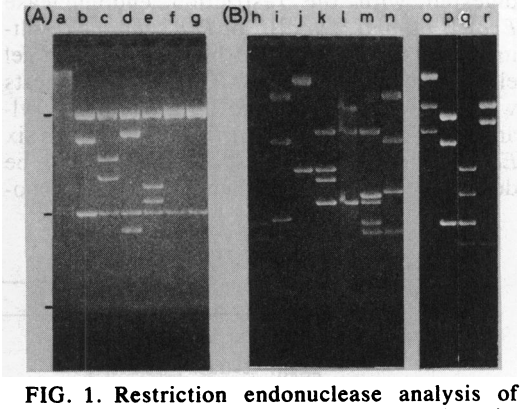

FIG. 1. Restriction endonuclease analysis of ColE1::Tn*2608* DNAs by agarose gel electrophoresis. (A) *Eco*RI-digested plasmid DNAs of (lane a) RGN823, (b) ColE1::Tn*2608*-1, (c) ColE1::Tn*2608*-2, (d) ColE1::Tn*2608*-3, (e) ColE1::Tn*2608*-4, (f) ColE1::Tn*2608*-5, and (g) ColE1::Tn*2608*-6. Small bars on the left represent common fragments of (from top to bottom) 7.80, 2.65, and 1.20 kb. (B) ColE1::Tn*2608*-1 DNA was digested with (lane i) *Hin*dIII and *Bam*HI, (j) *Bam*HI, (k) *Eco*RI and *Bam*HI, (l) *Eco*RI, (m) *Eco*RI and *Hin*dIII, (n) *Hin*dIII, (p) *Eco*RI, (q) *Eco*RI and *Pst*I, or (r) *Pst*I. Lanes h and o, *Hin*dIII-digested lambda phage DNA molecular size standard (23.7, 9.46, 6.67, 4.26, 2.25, and 1.96 kb, from top to bottom). All DNA digests were electrophoresed on a 0.8% agarose gel.

7.80, 2.65, and 1.20 kilobases (kb), which were also generated from the *Eco*RI-digested RGN823 DNA. Two more fragments of different molecular size were generated from each DNA digest of ColE1::Tn*2608*-2 (Fig. 1c), ColE1::Tn*2608*-3 (Fig. 1d), and ColE1::Tn*2608*-4 (Fig. 1e). From the DNA digests of ColE1::Tn*2608*-1, two more fragments of 5.60 and 2.65 kb were detected (Fig. 1b). This 2.65-kb fragment appeared as a doublet on the gel (the third fragment from the top). In ColE1::Tn*2608*-5 and ColE1::Tn*2608*-6, the transposition of Tn*2608* occurred into the colicin gene of the ColE1 plasmid, resulting in colicin nonproduction. The smallest fragment cleaved by the *Eco*RI site in the colicin gene and the outermost *Eco*RI site in Tn*2608* was too small to be detected under the agarose gel electrophoresis conditions used. Therefore, five fragments were generated from all types of ColE1::Tn*2608* plasmid by *Eco*RI digestion. The results indicate that development of Hg^r, Sm^r, Su^r, and $ColE1^r$ clones is due to the transposition of an identical DNA segment, Tn*2608*, in the ColE1 plasmid. Tn*2608* was also shown to be able to transpose from a ColE1::Tn*2608* plasmid to a conjugative plasmid, R388, in a *recA*-deficient bacteria (data not shown). The total DNA size of Tn*2608* is 13.5 kb.

To construct a restriction cleavage map of Tn*2608*, ColE1::Tn*2608*-1 DNA was completely digested with the restriction endonuclease *Eco*RI, *Bam*HI, *Hin*dIII, or *Pst*I, and the resulting digests were analyzed by 0.8% agarose gel electrophoresis (Fig. 1B). The *Eco*RI-digests were also electrophoresed on a 5% polyacrylamide gel (data not shown). This plasmid has six *Eco*RI sites (the smallest fragment could be detected on 5% polyacrylamide gel electrophoresis only), four *Hin*dIII sites, four *Pst*I sites, and two *Bam*HI sites. Since ColE1 has one *Eco*RI site and two *Pst*I sites, the Tn*2608* portion of ColE1::Tn*2608*-1 must contain five *Eco*RI sites, four *Hin*dIII sites, two *Pst*I sites, and two *Bam*HI sites. The relative locations of restriction cleavage sites were determined by a series of double digestion experiments (Fig. 1B). The final restriction cleavage map is presented in Fig. 2. The location of Tn*2608* in the ColE1::Tn*2608*-1 plasmid was determined through an electron microscopic analysis of heteroduplex molecules formed between ColE1::Tn*2608*-1 and pMK1 (data not shown).

The individual regions in Tn*2608* necessary for the expression of resistance to streptomycin, sulfonamide, and Hg were determined by cloning the fragments generated after digestion with *Hin*dIII, *Eco*RI, or *Pst*I. The genes for Hg resistance and sulfonamide resistance could not be cloned by digestion with *Eco*RI and *Pst*I, respectively, suggesting that each region necessary for expression of Hg and sulfonamide resistance must include an *Eco*RI or a *Pst*I site, respectively. The results are presented on the restriction cleavage map of Tn*2608* (Fig. 2 and Fig. 4)

Previously, the transposon Tn*21*, with a phenotype similar to Tn*2608*, was found to reside on plasmid R100 (12). To obtain a restriction map of Tn*21*, Tn*21* was transposed from YC1130, a *recA* derivative of CRT46 Rif^r Hfr (R100-1) constructed by M. Yoshikawa, to a conjugative plasmid R388 (data not shown). Restriction endonuclease cleavage analysis of the resulting plasmid R388::Tn*21* DNA was performed, and a structural map of Tn*21* was constructed (Fig. 4).

Identification of transposon Tn*2613* encoding Hg resistance. A new Hg resistance transposon

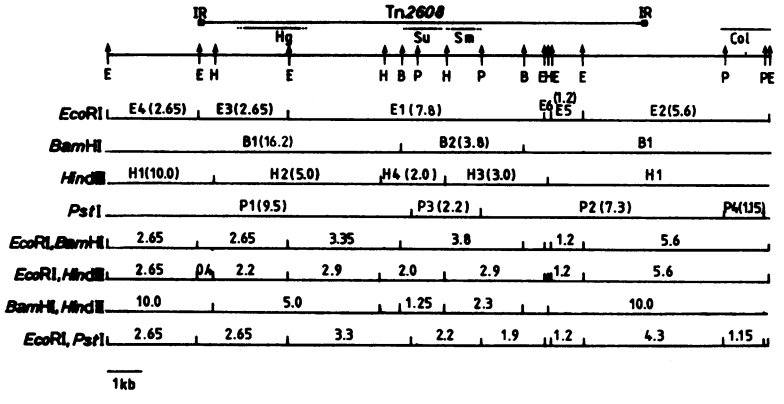

FIG. 2. Restriction endonuclease cleavage map of ColE1::Tn*2608*-1. The fragments were designated according to increasing migration in agarose gel electrophoresis patterns (Fig. 1B). Distances (in kilobases) between cleavage sites are indicated. Abbreviations: E, *Eco*RI; B, *Bam*HI; H, *Hin*dIII; P, *Pst*I; IR, inverted repeats; Sm, streptomycin; Su, sulfonamide; Hg, mercury; Col, colicin.

has been detected on a naturally occurring plasmid pCS229 by using a CRT46 Rif^r derivative with an integrated pCS229 (Yamamoto et al., in press). Plasmid DNA was prepared from the CRT46 Rif^r derivative with an integrated pCS229 and harboring pMK1. This DNA was used to transform competent C600 cells, which were then selected for Hg^r or Sm^r. Of 130 Sm^r transformants tested, all were resistant to Hg, streptomycin, sulfonamide, chloramphenicol, kanamycin, and ampicillin, suggesting that these carried autonomous pCS229 due to the excision from the integrated state. Of 130 Hg^r transformants tested, 119 clones also conferred all resistances mediated by pCS229. The remaining 11 transformants exhibited resistance to Hg and kanamycin, and plasmid DNAs prepared from three of these transformant clones were analyzed by 0.8% agarose gel electrophoresis after digestion with *Eco*RI (data not shown). All DNA had three common fragments of 2.65, 1.65, and 1.20 kb, and two more fragments of different sizes, suggesting that the common sequence encoding Hg resistance transposed to various sites of the pMK1 plasmid. We designated this transposable element Tn*2613*. The transposability of the Sm^r gene on pCS229 was not detected, although an intensive study was performed. Next, an endonuclease cleavage map of pMK1::Tn*2613*-2 was constructed. pMK1::Tn*2613*-2 DNA was completely digested with *Eco*RI, *Bam*HI, *Hin*dIII, or *Pst*I, and the resulting digests were electrophoresed on 0.8% agarose gel (data not shown). This plasmid had six *Pst*I sites, five *Eco*RI sites, four *Hin*dIII sites, and one *Bam*HI site, of which four *Eco*RI sites and two *Hin*dIII sites were assigned to Tn*2613* because six *Pst*I sites, two *Hin*dIII sites, one *Eco*RI site, and one *Bam*HI site were included in pMK1 plasmid. The final restriction cleavage map of pMK1::Tn*2613*-2 was constructed (Fig. 3) on the basis of a double-digestion experiment with *Eco*RI and *Hin*dIII, a comparison of the cleavage patterns of pMK1, whose cleavage map has been constructed (24), and a heteroduplex analysis between pMK1::Tn*2613*-2 and ColE1 DNAs (data not shown). The region required for the expression of Hg resistance was also located through a cloning experiment by *Eco*RI or *Hin*dIII digestion of pMK1::Tn*2613*-2 and mapped on the final restriction cleavage map of Tn*2613* (Fig. 3 and Fig. 4).

Comparison of physical and functional characteristics of various transposons determining multiple resistance. The characteristics of Tn*2608*, Tn*2613*, and related transposons are summarized in Table 1, and their physical and functional maps are shown in Fig. 4. Of these, Tn*2613* and Tn*501* encode a similar phenotype, Hg resistance, their difference occurring in the region of the right-hand side of each transposon. This region in Tn*501* encodes the *tnpA* and *tnpR* genes necessary for its normal transposition (20). Tn*2608* and Tn*21*, though distinctly different in size, specify a similar phenotype, streptomycin, sulfonamide, and Hg resistance. Tn*2607*, which has been found recently on a conjugative plasmid RGN14 (6; unpublished data), and Tn*4* include, respectively, in their structures, Tn*2601* (23) and Tn*3* (13), which are ampicillin transposons specifying the type I β-lactamase. Tn*2603*-mediated resistance to ampicillin is associated with production of the type II β-lactamase (oxacillin-hydrolyzing), and its genetic and physical fine structures have been already established (24).

On the basis of these physical and functional maps, the evolutionary route leading to the formation of related transposons from a common ancestral mercury transposon is hypothesized below. It appears that the mercury transposon Tn*2613* somehow acquired the DNA sequence encoding streptomycin and sulfonamide resistance, leading to the generation of a transposon mediating streptomycin, sulfonamide, and Hg resistance, Tn*2608*. The mechanism for inserting this DNA sequence into the middle of

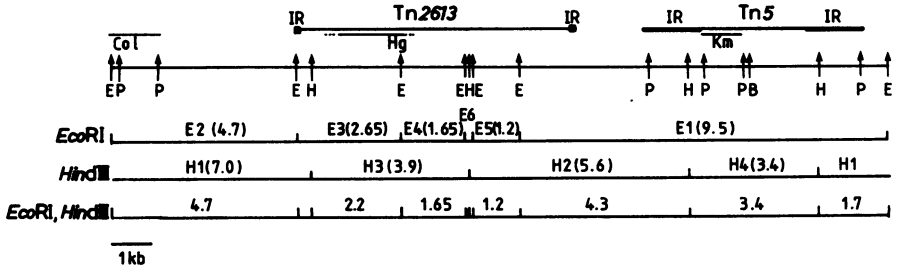

FIG. 3. Restriction endonuclease cleavage map of pMK1::Tn*2613*-2. The fragments were designated according to increasing migration in agarose gel electrophoresis (Fig. 2B). Distances (in kilobases) between cleavage sites are indicated. Abbreviations: see the legend to Fig. 2; Km, kanamycin.

TABLE 1. Properties of multiple-resistance transposons

Transposon	Original plasmid	Resistance phenotype	Total size (kb)	Source (reference)
Tn*501*	pVS1	Hg^r	8.2	P. M. Bennett (3)
Tn*2613*	pCS229	Hg^r	7.2	This study
Tn*2608*	RGN823	Hg^r Sm^r Su^r	13.5	This study
Tn*21*	R100	Hg^r Sm^r Su^r	19.0	This study, D. J. Kopecko (12)
Tn*2603*	RGN238	Hg^r Sm^r Su^r Ap^r	20.0	(25)
Tn*2607*	RGN14	Sm^r Su^r Ap^r	24.0	This study
Tn*4*	R1	Sm^r Su^r Ap^r	22.5	D. J. Kopecko (13)

Tn*2613* is unknown at present because the streptomycin and sulfonamide resistance genes were not independently transposable. The structural features of Tn*2613* are strictly conserved in Tn*2608*, suggesting a close evolutionary relationship between them. Next, the insertion of a DNA sequence of about 5.5 kb, of unknown phenotype, into the central portion of Tn*2608* seemed to be involved in the formation of Tn*21*. In addition to the insertion, Tn*21* contains an apparent minor sequence alteration in the left inverted repeat segment and in the right portion relative to Tn*2608*. A further step could have been the acquisition of the DNA sequence specifying the type II β-lactamase gene by Tn*21*, leading to the generation of Tn*2603*. Heteroduplex analysis between Tn*2603* and Tn*4*, being closely related to Tn*21* (Fig. 5), indicates that the substitution between DNA sequences might have been involved in the aquisition of the type II β-lactamase gene by Tn*21*.

An alternative route in this evolution could have been the transposition of ampicillin transposons Tn*2601* and Tn*3*, into the Hg resistance operon of Tn*21*, leading to the generation of Tn*2607* and Tn*4*, respectively. These transpositions seem to have caused the inactivation of the expression of Hg resistance. Heteroduplex analysis shown in Fig. 5 suggests that a 1.5-kb DNA sequence has been deleted in Tn*4*.

Our hypothesis for the evolution of multiple-resistance transposons from an ancestral mercury transposon implies that the functions required for their transposition are essentially involved in a mercury transposon. Of the related transposons, Tn*2603* and Tn*501* have been already elucidated with respect to the genes necessary for their transposition, *tnpA* and *tnpR* (20, 22).

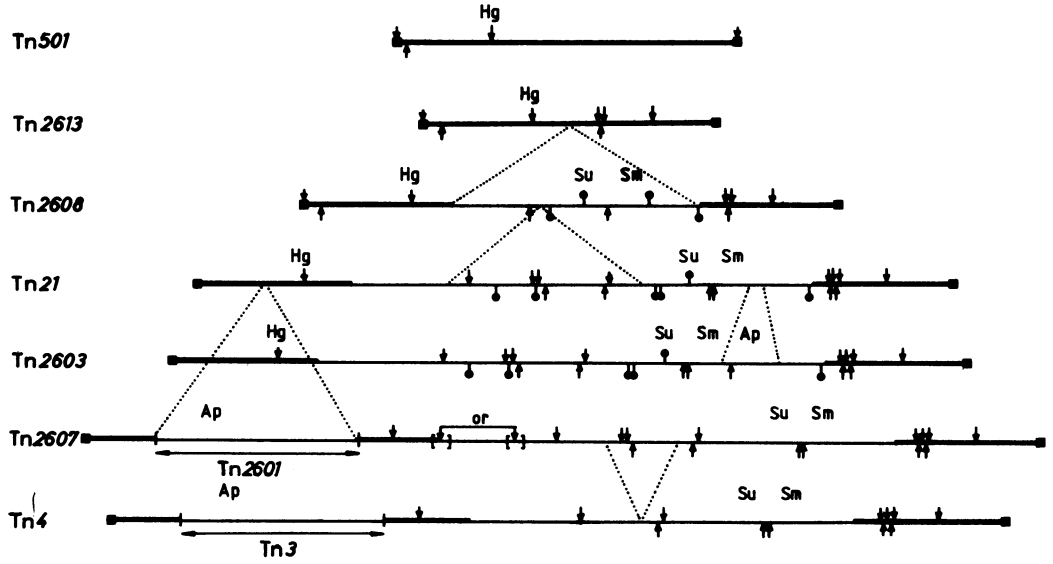

FIG. 4. Physical and functional maps of related resistance transposons. Heavy lines indicate the DNA region homologous to that of Tn*2613*. The cleavage sites of *Bam*HI and *Pst*I in Tn*2607* and Tn*4* are unknown. The map of Tn*501* is from Bennett et al. (3) and that of Tn*4* is from Clerget et al. (5). Abbreviations: see legend to Fig. 2; Ap, ampicillin. Symbols: ■, inverted repeat segment; ↓, *Eco*RI site, ↑, *Hin*dIII site; ↓, *Bam*HI site; ↑, *Pst*I site.

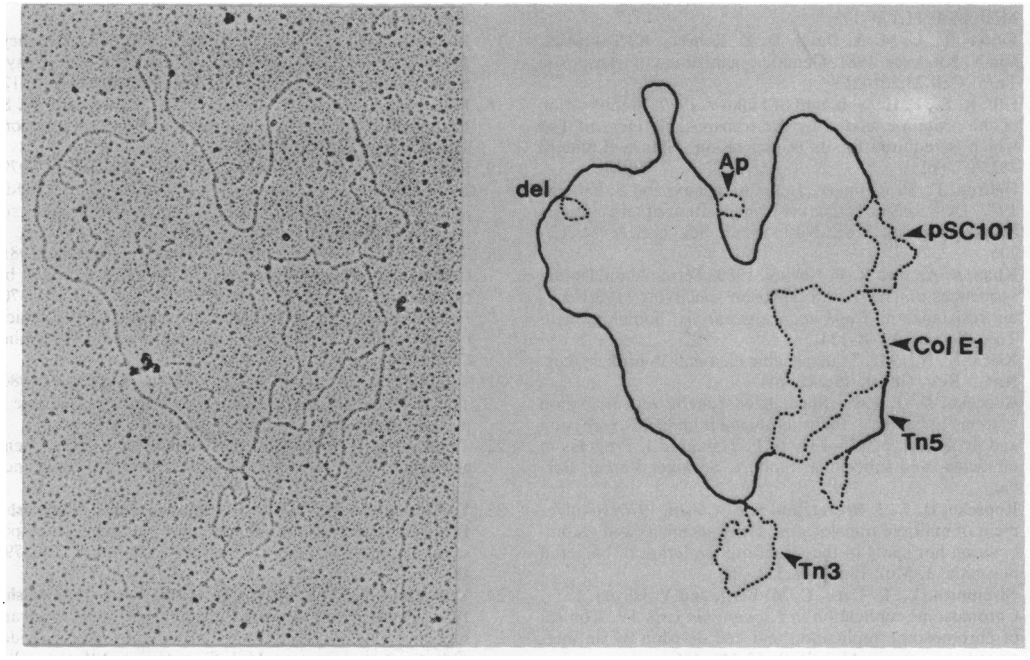

FIG. 5. Heteroduplex analysis between Tn*2603* and Tn*4* DNAs. A heteroduplex was formed between pMK1::Tn*2603* and pSC101::Tn*4*. Solid lines and dotted lines represent the double-stranded DNA and single-stranded DNA, respectively. Abbreviations: Ap, region specifying the type II β-lactamase gene; del, deleted region.

The functions of the genes seemed to be very similar to those of ampicillin transposons Tn*3* (4, 8) and Tn*2601* (26) and tetracycline transposon Tn*1721* (20), although the complementation for transposition among these transposons has not yet been revealed. It has been found that the DNA sequences of the terminal inverted repeat segments of Tn*3*, Tn*501*, and Tn*1721* are very similar (10). These results suggest that these transposons might have evolved from a common ancestor.

To date there have been at least five naturally occurring elements transposing the Hg resistance determinant: Tn*501*, Tn*2613*, Tn*3402* from *Citrobacter* spp. (17), Tn*3403* from *Klebsiella* spp. (17), and a transposon on R826 from *E. coli* (21). Tn*501* and Tn*2613* should be included, although partly different in structure, in an identical transposon family. However, the other mercury transposons described above are distinctly different from Tn*501* and Tn*2613* in size and structure (3, 17, 21). This is distinct from the ampicillin transposon family represented by Tn*3*, whose structures and functions are extremely identical. A mercury transposon family represented by Tn*2613* could have been highly evolved, leading to the generation of various multiple-resistance transposons through a dynamic alteration of the DNA sequence as shown in the present results. These may suggest that a mercury transposon was generated before the establishment of an ampicillin transposon or that there is strong pressure to make or keep the sequence identical in the ampicillin transposon.

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