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Tn*5253*, the Pneumococcal Ω(*cat tet*) BM6001 Element, Is a Composite Structure of Two Conjugative Transposons, Tn*5251* and Tn*5252*

PATRICIA AYOUBI, ALI O. KILIC, AND MOSES N. VIJAYAKUMAR*

Department of Microbiology, Oklahoma State University, Stillwater, Oklahoma 74078

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Tn*5253*, carrying tetracycline and chloramphenicol resistance determinants, is a 65.5-kb conjugative transposon originally detected in the chromosome of *Streptococcus pneumoniae* BM6001. We have identified an 18-kb segment of DNA carrying the *tet* determinant within Tn*5253* to be an independent conjugative transposon when removed from the context of the larger element. In vivo deletion of this DNA segment, now termed Tn*5251*, from within Tn*5253* did not affect the conjugative transposition properties of the remaining sequences. Thus, Tn*5253* is a composite element of two conjugative structures: Tn*5252*, constituting the sequences beyond Tn*5251* within Tn*5253*, and Tn*5251*. The transfer properties of Tn*5252* and Tn*5251* suggest that these may belong to two different classes of mobile elements even though they were initially found associated. The notion that a *tet*-carrying transposon like Tn*5251* may have been the ancestral element in the evolution of the larger streptococcal conjugative transposons must be reevaluated in the light of present observations.

Even though pneumococci (*Streptococcus pneumoniae*) can accept and maintain a variety of plasmids from other streptococci, for reasons that are not readily apparent endogenous extrachromosomal elements have only rarely been observed in this species. The recent worldwide emergence of multiple antibiotic resistance in this severe human pathogen (3, 9, 16) has been associated with a novel class of genetic elements, termed conjugative transposons. Localization of Tn*916* (Tc^r) in *Enterococcus faecalis* DS16 (4, 10), Tn*1545* (Tc^r Em^r Km^r) in *S. pneumoniae* BM4200 (6, 7, 12), Tn*3701* (Tc^r) in *S. pyogenes* A454 (19), Tn*3951* (Tc^r Em^r Cm^r) in *S. agalactiae* B109 (16, 17, 32), and others (15) attests to the widespread dissemination of this group of elements among streptococci. Conjugative transposons transfer among a number of streptococci by a process that is independent of host recombination systems (10) and requires cell-to-cell contact (31).

Over the last decade, substantial progress has been made toward the genetic and molecular characterization of these mobile elements, which range from about 16 to about 68 kb in size. On the basis of size, these conjugative elements could be loosely classified into two types, one ranging from 16 to 25 kb and the other being about 60 kb. Besides transfer functions, each transposon may also carry one or more antibiotic resistance determinants, and a common feature among most if not all of these is the presence of a homologous tetracycline resistance determinant (33) of the type *tetM* (2). Other antibiotic resistance determinants include Cm^r, Km^r, Em^r, and Sm^r. The prevalance of a *tet* determinant of type M and extensive homology between regions of DNA surrounding this gene among most of the conjugative transposons suggested that a smaller conjugative element such as *tet* carrying Tn*916* (16 kb) could have served as a progenitor in the evolution of the larger elements (5, 13). Autoaccumulation of other heterologous elements carrying a variety of determinants could have resulted in the observed increase in size. A further implication of this speculation is that the conjugative transfer of all these elements shares a common mechanism, as the elements are derived from an ancestral element with transfer properties.

Tn*5253*, formerly called the Ω(*cat tet*) element (36), was originally detected as a heterologous insertion in the chromosome of the plasmid-free clinical isolate *S. pneumoniae* BM6001 (3, 9). By inserting the *Escherichia coli* vector plasmid pVA891 (22) (which is incapable of autonomous replication in streptococci) at many sites specifically within Tn*5253*, we were able to clone and recover parts of the element in *E. coli* (37). Physical analysis of the passenger DNAs from these plasmids made it possible to construct a detailed restriction map of this 65.5-kb element, to localize the drug resistance determinants, and to identify its junction and target regions in the pneumococcal chromosome (36), (Fig. 1). With the cloned fragments derived from Tn*5253*, we sought to investigate whether a Tn*916*-like element could have given rise to the larger conjugative transposons. Here we present evidence to show that Tn*5253* is indeed a composite element of two independent conjugative elements, the *tet*-carrying Tn*5251* that resembles the Tn*916* class of elements, and Tn*5252*, comprising sequences beyond Tn*5251*.

(A preliminary report of this work was presented at the Third International ASM Conference on Streptococcal Genetics, Minneapolis, Minn., 1990.)

MATERIALS AND METHODS

Bacterial strains, transformation, and conjugation. *S. pneumoniae* Rx1 is our standard laboratory strain (30), and DP1322 is Rx1 carrying Tn*5253* (30). DP1324 is DP1322 carrying the *str-1* chromosomal point mutation conferring resistance to streptomycin (Str^r). *S. pyogenes* ATCC 21547 was obtained from the Stillwater Medical Center. *S. agalactiae* ATCC 12386 and recombination-deficient, rifampin-resistant (Rif^r), and fusidic acid-resistant (Fus^r) *E. faecalis* UV202 (38) were used as recipients in some of the conjugation experiments. Growth of pneumococcal cultures, conjugation, competence regimen, and plating techniques have

* Corresponding author.

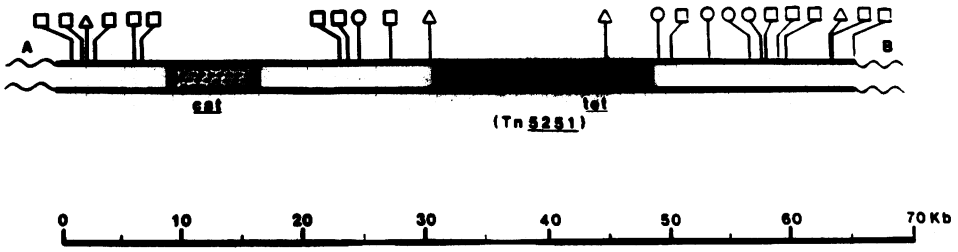

FIG. 1. Physical structure of Tn*5253*. Symbols: =, Tn*5253* DNA; ≈, pneumococcal DNA; A and B, *cat* and *tet* ends of the element respectively; ▦ *cat* region; ▯, direct repeats flanking the *cat* segment; ▨ *tet* region that transposes when removed from Tn*5253*; □, *Xba*I; △, *Kpn*I; ○, *Bam*HI.

been described previously (30, 31). All the streptococcal strains were grown without aeration at 37°C in CAT medium, a rich broth containing casein hydrolysate and tryptone (United States Biochemical Corp. and Difco). Conjugal transfer was enhanced when both donors and recipients were grown in Difco Casitone. Filter matings between pneumococci were limited to 4 h, whereas matings between pneumococci and *E. faecalis* were allowed 18 h at 37°C. Recombinant plasmids were generated in recombination-deficient *E. coli* DH1 or HB101 by transformation by the method of Hanahan (14).

DNA manipulation and analyses. Restriction endonucleases and T4 DNA ligase were purchased from Bethesda Research Laboratories and Promega. Enzyme reactions were carried out as recommended by the suppliers.

Chromosomal DNA from *S. pneumoniae* was prepared by the method of Marmur (24). Lysis and DNA isolation from *E. faecalis* have been described previously (10). Similar methods were used to isolate the chromosomal DNAs from *S. agalactiae* and *S. pyogenes*. *E. coli* isolates were screened for recombinant plasmids by agarose gel electrophoresis of rapid alkaline lysis products (1). For other purposes, plasmid DNA was isolated from *E. coli* by using standard cell lysis methods involving lysozyme treatment followed by sedimentation on cesium chloride-ethidium bromide density gradients.

Agarose gel electrophoresis and DNA purifications from agarose gels by electroelution were done essentially as described by Maniatis et al. (23). DNA hybridizations were done by the method of Southern (34) with the GeneScreen Plus membrane (Dupont, NEN) as support. Radioactive probe DNA was prepared by nick translation (23) of appropriate plasmids with [^{32}P]dCTP (Dupont, NEN).

RESULTS

Instability of the *tet* element in *E. coli*. If the larger streptococcal conjugative elements evolved by the addition of several genetic units into a *tet*-carrying prototype element such as Tn*916*, the termini of all the conjugative transposons would be expected to carry significant homology. To test whether such was the case, we used the plasmid pAM118 (11), carrying the entire Tn*916* on the vector plasmid pVA838 (22), as a probe in blot hybridization experiments with digests of DP1322 DNA carrying Tn*5253*. However, pAM118 failed to hybridize to either of the termini of Tn*5253*. The homology within Tn*5253* to Tn*916* was confined to the region containing *tet* in one contiguous segment (data not shown).

Furthermore, when a 23-kb *Xba*I fragment containing the *tet* region from Tn*5253* was cloned into pVA891, the resulting plasmid, pVJ403, was stable in *E. coli* when tetracycline selection was maintained. However, when the tetracycline-resistant *E. coli* cells were grown overnight in the absence of selective pressure, tetracycline-sensitive cells arose at a detectable frequency. Physical analysis of plasmids from a number of tetracycline-sensitive isolates showed that in each case the loss of tetracycline resistance was associated with the deletion of an 18-kb segment of DNA internal to the passenger portion of the parental plasmid. The restriction maps of the parental plasmid, pVJ403, and a representative deletion-derivative plasmid, pVJ403Δ*tet*, are shown in Fig. 2. The deletion was mapped to lie between the *Hin*dIII site 3 kb away from the left end of the passenger DNA and the *Bam*HI site 1.4 kb from the right end. The resulting *Hin*dIII-*Bam*HI fusion fragment in all the isolates was 0.8 kb, suggesting at least near-precise excision of the *tet* segment. It is worth pointing out that the excision of a related 25-kb conjugative transposon, Tn*1545*, from a plasmid replicon and transposition into the *E. coli* chromosome had been shown to occur under similar conditions, (11), which suggested that the excision of the *tet* segment from pVJ403 could also have been related to its transposition.

Transposition of the *tet* element from pVJ403 in pneumococci. Although the excision of the *tet* element in *E. coli* seemed to indicate transposition, there has been no report of independent movement of this segment of DNA when present in pneumococcal chromosome as a part of the larger Tn*5253*. To determine whether the *tet* element was capable of transposition when removed from the Tn*5253* context, we introduced pVJ403 into wild-type *S. pneumoniae* Rx1 cells

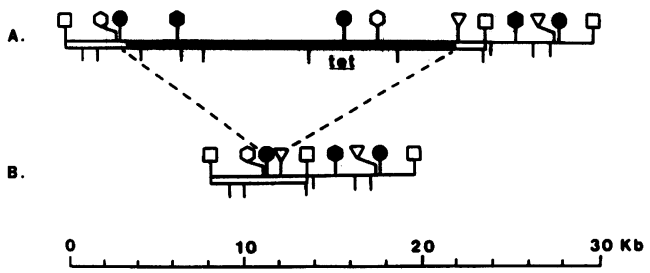

FIG. 2. Restriction endonuclease map of the *E. coli* plasmid pVJ403 and the deletion derivative pVJ403Δ*tet*. (A) pVJ403; (B) pVJ403Δ*tet*. Symbols: —, the vector (pVA891) portion; □, passenger DNA derived from Tn*5253* (see text); □, *Xba*I; ●, *Kpn*I; △, *Bam*HI; ●, *Hin*dIII; ○, *Eco*RI; | (below line) *Hin*cII; ▬, 18-kb Tn*5251* that excises in *E. coli* in the absence of tetracycline selection. The ends of Tn*5251* were determined from DNA sequencing studies (not given). The location of the *tet* determinant is indicated.

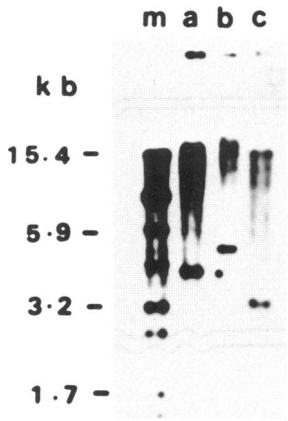

FIG. 3. Autoradiogram showing random insertion of Tn*5251* from the *E. coli* plasmid pVJ403 into the pneumococcal chromosome during transformation. Genomic DNAs from three Tc^r transformants (lanes a through c) were digested with *Eco*RI, resolved on a 0.8% agarose gel, transferred to a nylon membrane, and hybridized to [^{32}P]dCTP-labeled pVJ403. Tn*5251* carries a unique site for *Eco*RI (Fig. 2). Lane m contains marker DNA, consisting of a set of calibrated fragments from pVA891 or derivative plasmids (36), all of which react with the probe. Representative sizes are indicated.

via transformation. As only one of the strands of a bound donor molecule is taken up by a cell during pneumococcal transformation (18), plasmid establishment would require entry of two overlapping complementary molecules, replicative filling in of the gaps, and subsequent generation of an intact circle (27). Since the vector portion (derived from pVA891) in pVJ403 was incapable of autonomous replication in streptococci, we did not expect any Em^r transformants, and we found none. As the wild-type Rx1 genome did not carry homology to any portion of the passenger DNA in pVJ403, homologous genetic recombination by the normal pathway was not expected. However, about 50 Tc^r transformants per 2×10^7 CFU per 10 μg of plasmid DNA were obtained. All were sensitive to erythromycin, indicating that the vector was lost. The use of chromosomal DNAs from several of these clones as donors in a second round of transformation of Rx1 cells increased the number of Tc^r transformants observed by 4 orders of magnitude. This increase in transformation efficiency as a result of the homology provided by the flanking sequences indicated the insertion of the *tet* into the chromosome. Because of the absence of homology between the recipient chromosome and the donor plasmid DNA used in the initial transformation, the insertion of the heterologous *tet* marker could not have involved the homology-dependent insertion-duplication pathway (35). Hence, it was likely that the integration of *tet* into the chromosome was the result of transposition.

To determine whether the integration of the *tet* segment during transformation involved unique or multiple target sites, we analyzed chromosomal DNAs from several Tc^r transformants in blot hybridization experiments with pVJ403 and pVJ403Δ*tet* as probes. That the insertion of the *tet* segment did not involve any homologous pairing of sequences beyond the 18-kb transposing segment in pVJ403 was evident as pVJ403Δ*tet* did not react with any of the samples (data not shown). On the other hand, pVJ403 strongly hybridized to at least two fragments representing chromosome-*tet* element junction regions in each case (Fig. 3). The differences in sizes of the junction fragments in different clones indicated random insertion of the element and ruled out the possibility of any plasmid forms.

Furthermore, three Tc^r clones were used as donors in filter-mating experiments with Rx1 recipients to test whether *tet* could be conjugally transferred from these transformants. Two were able to transfer *tet* at a frequency of 3×10^{-5} per donor under conditions where transfer of the chromosomal marker, *str*, could not be detected. From these results, it was clear that the *tet* determinant was within a transposon that was capable of conjugal transfer when removed from the larger element; this segment of DNA is now termed Tn*5251*. The restriction map of this 18-kb transposon (Fig. 2) showed significant similarities to that of the 16-kb transposon, Tn*916* (29), suggesting possible common ancestry.

In vivo deletion of Tn*5251* from Tn*5253*. The identification of Tn*5251* within the larger Tn*5253* raised questions regarding the functional role of the sequences beyond the smaller transposon but within the larger one. Is it possible that conjugal transfer of Tn*5253* was due only to the presence of the internal Tn*5251* with altered specificities? This possibility was considered less likely because of the differences in target selection between Tn*5251* and Tn*5253*. Tn*5253* has been shown to insert preferentially at a unique site in the pneumococcal genome (36), whereas Tn*5251* inserts randomly at many sites (see above). In any event, if the transfer of the entire Tn*5253* during conjugation was due to the presence of Tn*5251*, Tn*5253* devoid of Tn*5251* would be transfer deficient. To address these questions, we used the strategy shown in Fig. 4 to induce the deletion of Tn*5251* from within Tn*5253*, by using the plasmid pVJ403Δ*tet*. As mentioned above, Tn*5251* was excised and lost from pVJ403 when propagated in *E. coli* without tetracycline selection, giving rise to the deletion derivative pVJ403Δ*tet*. This derivative was digested with *Xba*I, and the 5.2-kb fusion fragment was electroeluted from an agarose gel and fed to competent DP1324 cells carrying the entire Tn*5253*. It was expected that as a result of the pairing between the donor molecule and the homologous sequences flanking Tn*5251* in the recipient, Tn*5251* would be looped out and lost in one of the strands and the heteroduplex would be resolved after one round of replication. It should be noted that in pneumococcal transformation a donor molecule rendered single stranded upon entry could introduce either deletion or insertion of a DNA segment into the chromosome as long as the region of alteration is flanked on either side by a sufficient length of homologous DNA to allow efficient register. After allowing 2 h for phenotypic expression in liquid broth, we plated the transformants on nonselective medium. The following day, 4,000 colonies were replica plated prior to screening for Tc^s and Cm^r transformants. Thirty Tc^s clones were found.

Chromosomal DNAs from four of these were digested with either *Eco*RI or *Hin*dIII, separated on an agarose gel, transferred to a nitrocellulose filter membrane, and probed with ^{32}P-labeled pVJ403 to determine whether they carried the intended deletion of Tn*5251*. The probe did not react with the DNA from the wild-type Rx1 cells, which did not carry Tn*5253* (Fig. 5). Owing to the presence of a single site, *Eco*RI cleaves the region of Tn*5251* within Tn*5253* into two fragments of 23 and 12.2 kb (36). As expected, the probe reacted with similar-sized fragments of DNA from DP1324 carrying the entire Tn*5253* transposon. Furthermore, three *Hin*dIII fragments of 12.6, 8.5, and 3.6 kb of DP1324 DNA reacted with the probe. However, the probe hybridized only with a single (17-kb) *Eco*RI fragment and two (3.6- and 3.1-kb) *Hin*dIII fragments of the chromosomes of the Tc^s

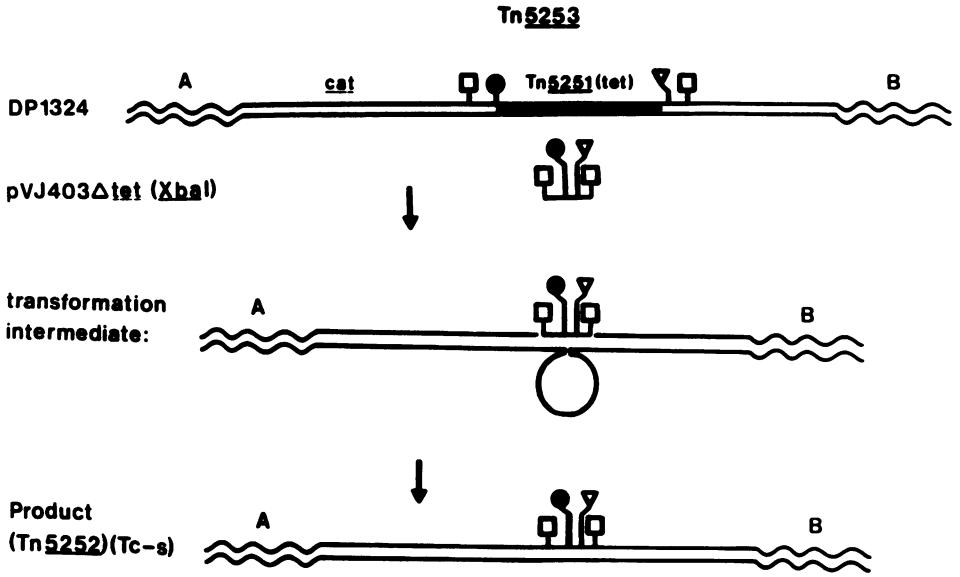

FIG. 4. Strategy for deleting Tn*5251* from Tn*5253*. The relevant restriction sites are shown. Symbols: □, *Xba*I; ●, *Hin*dIII; ▽, *Bam*HI. The passenger DNA (the 5-kb fusion fragment carrying sequences flanking Tn*5251* in Tn*5253*) from pVJ403Δ*tet* was isolated and used as donor DNA in transformation of DP1324. The donor molecule taken up as a single strand was expected to displace the resident strand and pair with the complementary strand, inducing the intervening segment containing Tn*5251* to loop out. After one round of replication and segregation of markers, Tc^s transformants arose (Tc-s). Tn*5253* DNA (——) and flanking chromosomal DNA (∿∿∿) are shown.

clones, indicating the deletion of the 18-kb Tn*5251* from within Tn*5253* in each case. One of these Cm^r Tc^s clones was designated SP1000.

Conjugative transfer of Tn*5252*. To determine whether SP1000 cells, carrying only the sequences beyond Tn*5251* within Tn*5253*, were capable of conjugal transfer, we used them as donors in filter-mating experiments with Rx1 recipients. Transconjugants were selected on plates containing chloramphenicol. The Cm^r marker transferred at a frequency of 10^{-6} to 10^{-7} per donor, which was comparable to that of the intact parental Tn*5253*. Because the sequences beyond Tn*5251* within Tn*5253* as in SP1000 were found to be capable of conjugal transfer by themselves, this segment of DNA was termed Tn*5252*.

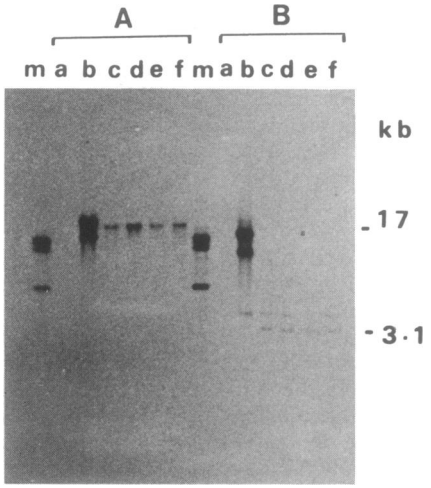

FIG. 5. Physical analysis of Tc^s transformants carrying the deletion of Tn*5251* from within Tn*5253*. Autoradiogram showing DNA-DNA blot hybridization of ^{32}P-labeled pVJ403 to (A) *Eco*RI and (B) *Hin*dIII digests of DNA from Rx1 (lanes a), DP1322 carrying Tn*5253*, (lanes b), and four Tc^s transformants (lanes c through f). Lanes m, marker DNA.

To determine whether Tn*5252* was also capable of interspecific conjugal transfer, SP1000 cells were filter mated with *S. agalactiae* and the *rec*-deficient *E. faecalis* UV202 recipients. In parallel, DP1324 cells carrying the parental element Tn*5253* were also used as donors in control matings with these recipients. In all cases, Cm^r transconjugants arose at a frequency of 10^{-5} to 10^{-6} per donor.

Next, to confirm the integrity of the transferred element in the *E. faecalis* transconjugants, we probed chromosomal DNAs from these transconjugants with the following plasmids in blot hybridization experiments: (i) pVJ407, carrying the 2.75-kb *Kpn*I fragment (containing the right junction region of Tn*5253*, as in DP1322) on the vector plasmid pUC19; (ii) pDP63, carrying the 3.6-kb *Eco*RI fragment (containing the left junction region of Tn*5253*, as in DP1322) on the vector plasmid pACYC184; and (iii) pVJ403Δ*tet*. The passenger DNAs in pVJ407 as well as pDP63 strongly reacted with DNA from each transconjugant under conditions where neither hybridized to the recipient chromosome (not shown), indicating that the transferred DNA contained both termini and hence possibly everything between them. Further confirmation of this was obtained when pVJ403Δ*tet*, carrying DNA from the middle section of the conjugative element, was also used as a probe in similar experiments (data not shown) and the resulting fragments were of the expected sizes. The fragments hybridizing to each junction probe were of the same size in five of the six Tn*5252* transconjugants, whereas one of the transconjugants seemed to have junction fragments of a different size, suggesting insertion at a different site.

Interestingly, each probe, pVJ407 and pDP63, hybridized to two restriction fragments of the Tn*5253* transconjugants, whereas they reacted with only one fragment of Tn*5252* transconjugants. The results of such an experiment in which

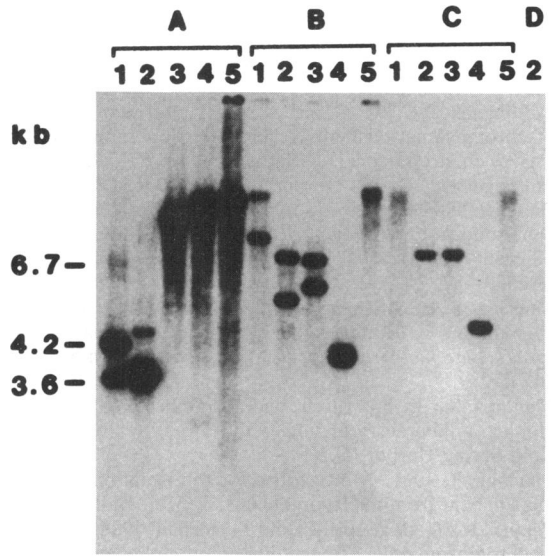

FIG. 6. Interspecific transfer of Tn*5252* from pneumococcal donors into *E. faecalis* UV202 recipients following conjugal mating. Autoradiogram showing DNA-DNA hybridization of the ^{32}P-labeled pDP63 carrying the left junction region of Tn*5253* as it is in the pneumococcal strain, DP1322, to restriction endonuclease-digested chromosomal DNAs from (A) DP1322, (B) an *E. faecalis* Cm^r Tc^r transconjugant from mating with DP1322, (C) an *E. faecalis* Cm^r transconjugant from mating with SP1000 carrying Tn*5252*, and (D) the recipient, *E. faecalis* UV202. Lanes: 1, *Bgl*II; 2, *Eco*RI; 3, *Hin*dIII; 4, *Kpn*I; 5, *Pst*I.

a representative from each class of transconjugants was used are shown in Fig. 6. Whether the two copies of Tn*5253* in the *E. faecalis* transconjugants represented tandem repeats or other forms could not be determined.

DISCUSSION

This investigation was initiated to gain some understanding of the evolution of conjugative transposons, which have been largely responsible for the sudden emergence and horizontal spread of multiple antibiotic resistance among clinical streptococci. Almost all these elements identified so far share substantial homology, at least around the *tet* region, suggesting common ancestry. On the basis of this observation, a smaller conjugative transposon such as Tn*916* was considered to be a possible prototype element. However, the results presented in this report demonstrate that the *tet* region in Tn*5253* that is homologous to the Tn*916* class of transposons is by itself an independent conjugative transposon. This smaller element, Tn*5251*, displayed conjugal properties when removed from the larger element, Tn*5253*. In support of our findings, Le Bouguénec et al. also have identified a transposable element, Tn*3703*, present within the larger (>50-kb) conjugative transposon, Tn*3701*, carried by *S. pyogenes* A454 (19–21).

Besides the identification of Tn*5251*, the other significant finding in this work was that Tn*5252*, which is made up of the sequences beyong Tn*5251* within Tn*5253*, was capable of conjugative transposition. Tn*5252* seemed to insert at more than one site following conjugal transfer in *E. faecalis*. The transfer of this element into *E. faecalis* UV202 attests to its independence from host recombination functions, rendering this class of elements distinct from the *E. coli* F-like plasmids.

Tn*5252* seemed to prefer to insert at a unique target site in pneumococci. This behavior was similar to that of the parental element, Tn*5253*, and was different from that of Tn*5251*, indicating that Tn*5251* probably did not play a mechanistic role in the conjugation of the larger element. Hybridization studies done in this and other laboratories (20) suggest that Tn*5251* was closely related to the Tn*916*, Tn*1545*, and Tn*3703* class of transposons and structurally distinct from the Tn*5252* class of elements. Besides, the observed differences between the two types of elements in their target selection following conjugal transfer may be due to different modes of transfer (26, 36). Convincing evidence of a circular intermediate in the transposition of Tn*916* has been obtained (28), and it is likely that all the smaller conjugative transposons belonging to the Tn*916* class of elements function similarly. However, the lack of restriction of Tn*5253*, while simultaneously entering plasmids were being restricted, during conjugal transfer into recipients carrying the *Dpn*II system (13) suggests the possibility that the mechanism of transfer of the larger conjugative transposons may differ in details from those of the Tn*916* class of elements. Our results would be consistent with the previous observations (20) and point to the presence of two classes of conjugative transposons in streptococci, the smaller ones typified by Tn*916* (29) and the larger ones by Tn*5252*. Implicit in these results is the conclusion that smaller elements such as Tn*5251* were added at a later date to larger ones like Tn*5252* to create composite structures; hence, Tn*916*-like transposons could not be considered progenitors of the larger elements. In agreement with Le Bouguénec et al. (20), our results seem to suggest that a Tn*5252*-like element should be considered a prototype of the composite conjugative elements such as Tn*5253*.

Even though Tn*5251* and Tn*5252* are both capable of independent conjugal transfer, separation of these elements has not been observed when they are associated as Tn*5253*. It is possible that the inability of Tn*5251* to move independently from within Tn*5253* is due to the neighboring flanking sequences. On the other hand, interruption of a region 2 kb away from the right end of Tn*5251* within Tn*5253* repeatedly induced the excision of the smaller *tet* element (37), suggesting a possible interaction between the two.

The origin and composition of the larger conjugative transposons may turn out to be somewhat complicated, as indicated by the presence of Tn*5251* and the two insertion sequence-like elements within Tn*5253* (36). The *cat* region flanked by direct repeats in Tn*5253* has been shown to be homologous to the staphylococcal plasmid pC194 (25). Further work may reveal whether there is a propensity for the autoaccumulation of various genetic units into prototype elements such as Tn*5252* and Tn*3701* to form larger conjugative structures. Without the 18-kb Tn*5251* and 7.5-kb *cat* segment, the remaining portion of DNA of Tn*5253* constituting Tn*5252* is about 40 kb. Localization of transfer-related and other genes within Tn*5252* may provide further insight into the nature of this interesting element.

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