GENETIC CHARACTERIZATION OF A COMPOSITE STREPTOCOCCAL CONJUGATIVE TRANSPOSON, TN*5253*

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CHAPTER I

INTRODUCTION

Transposable elements are probably one of the most powerful tools in the evolution of the genetic make-up of organisms. They are found in various bacteria and eukaryotic organisms. They can result in large scale rearrangement of adjacent DNA sequences following their insertion in the host replicon. Moreover, mobile DNA elements often carry drug resistance genes which are of medical concern.

Until recently, drug resistance among the clinical isolates of streptococci was found to be plasmid associated. However, when multiple-antibiotic resistant clinical isolates of *Streptococcus pneumoniae* began to appear worldwide in the 1970s, efforts by several research groups to isolate R-plasmids from these strains were unsuccessful (31, 58, 71, 84). The genetic elements mediating multiple-antibiotic resistance and exhibiting unique properties of intracellular and intercellular transposition have been designated "conjugative transposons" and found to be associated with the chromosome of streptococci (6, 8, 25, 31, 37, 39). A number of conjugative transposons have been subsequently reported in clinical isolates of pneumococci, and in groups A, B, F, D, and G streptococci (8, 26). Most of these streptococcal conjugative elements carry a tetracycline-resistance (Tc^r) determinant, either alone or linked to chloramphenicol (Cm^r), erythromycin (MLS^r) or kanamycin (Km^r) resistances in various combinations.

The studies discussed in this dissertation focus on the Ω(*cat tet*) BM6001 element isolated from the clinical strain, *S. pneumoniae* BM6001 (66). The Ω (*cat tet*) element (now termed Tn*5253*) is a 65.5-kilobase (kb) conjugative transposon carrying resistance to chloramphenicol and tetracycline.

By using an *E. coli* plasmid, pVA891 (45), as a tool in directed insertion mutagenesis, Vijayakumar and coworkers (76, 77) were able to clone various segments of transposon DNA and obtain a detailed restriction map of the BM6001 element with eleven different restriction enzymes. The termini, target and drug resistance determinants of the element were also localized and cloned in *E. coli.*

The presence of a tetracycline-resistance determinant in all streptococcal conjugative transposons raised the possibility that a Tn*916*-like element represented a prototype of these elements and served as a receptacle for the accumulation of other drug resistance genes resulting in an increase in size (18, 31). However, the data from DNA-DNA hybridization experiments did not support this hypothesis (43). If a drug resistance gene was added to a preexisting Tn*916*-like progenitor element, the termini of the subsequent composite element would be expected to be homologous to the ends of Tn*916*. However, when it was used as a probe, Tn*916* hybridized only to the *tet* region of the Ω(*cat tet*) element and other composite transposons such as Tn*3701* and Tn*3951*, it did not bind to the temini of any of the composite transposons (43).

In addition, Tn*916*-like transposons transfer to streptococcal recipients and insert into recipient chromosome randomly whereas the larger elements like Tn*5253*, Tn*3701* and Tn*3951* seemed to insert at a single preferred target site on the recipient chromosome following conjugal transposition.

These lines of evidence strongly suggested that evolution of these elements was different than previously speculated.

The point of departure for this work was to understand the structural and functional properties of the Tn*916*-like sequence within the larger Ω(*cat tet)* element. When cloned into an *E. coli* vector, the Tn*916*-like sequence from the central region of Tn*5253* spontaneously excises in the absence of tetracycline selection. Similar observations have also been reported by other research groups with Tn*916* (27) and Tn*1545* (11) under the same conditions. Therefore, the questions to be addressed were whether the *tet* element was able to excise from the vector plasmid, pVJ403, in pneumococci and insert into the recipient chromosome. If so, would the excision of the *tet* element from the vector be precise and insertion random? Would the *tet* element be able to independently transfer among pneumococci by conjugation?

Another question was whether the conjugative transfer properties of Ω(*cat tet)* element were solely due to the presence of an internal Tn*916*-like sequence. Ayoubi was able to construct an *in vivo* deletion derivative of the Ω(*cat tet)* element devoid of the internal *tet* element (designated Tn*5251*) (3). This deletion strain (designated Tn*5252*) was shown to be capable of intraspecific and interspecific transfer without any mechanistic support of the Tn*916*-like sequence. In addition, functional similarities between Tn*5252* and parental transposon Tn*5253* showed that Tn*916*-like transposons and Tn*5252*-type transposons probably belong to two different classes of transposons.

In contast to previous speculation, it might be possible that the Tn*5252*-like larger elements are the prototype of a novel class of conjugative transposons because the physical and functional properties of

these elements are different from those of the smaller Tn*916*-type elements. Therefore, Tn*5252*-like elements might have served as receptacle for the accumulation of Tn*916*-like smaller elements at a later time and given rise to the larger composite transposons such as Tn*5253*, Tn*3701* and Tn*3951*.

Among these larger conjugative transposons, Tn*5253* was the first one identified as a composite structure of two independent conjugative transposon, Tn*5251* and Tn*5252* (3). When Tn*5251* and Tn*5252* are physically associated as in Tn*5253*, Tn*5251* has never been observed to separate itself from the parental element and transfer independently. Furthermore, the conjugal transfer properties and target specificity of Tn*5252* were found to be the same as those of Tn*5253* suggesting that Tn*5251* did not play any functional role in the tranfer of Tn*5253* or Tn*5252* but its own transfer functions may be suppressed by the surrounding Tn*5252* sequences.

Since Tn*5252* exhibits unique physical and functional features and represents a different class of conjugative transposons, I have focused my efforts on its genetic and molecular organization to better understand the nature and evolution of the conjugative transposons.

Unlike the Tn*916*-class of transposons, little is known about the conjugal transposition functions of Tn*5252*-like transposons.

The goal of this study was to determine the functional map of Tn*5252* by introducing a series of deletions and insertions within the transposon. An 8 kb region that contains the chloramphenicol resistance determinant of Tn*5252* is flanked by IS-like direct repeats of approximately 1.7 kb (55). Recombination between these two sequences often leads to spontaneous curing of the *cat* gene. The loss of the *cat* region does not impair the conjugal transfer of Tn*5252* (55). Therefore, the remaining 39 kb DNA

segment in Tn*5252* must involve the conjugal transposition functions of the transposon. Of the 39 kb segment of DNA, what is the minimum length of DNA that is required for the transfer of the element? In other words, are there regions between the different loci which are not involved in the transfer related functions? Localization of specific regions was expected to facilitate identification of the specific genes that may be involve in the transfer of the transposon. This in turn would enable the characterization of the genes which are involved in different functions such as excision, integration, communication between donor and recipient cells, intracellular transfer, and target selection by the conjugative transposons.

CHAPTER II

LITERATURE REVIEW

The genus streptococci represent a diverse group of gram-positive species. Most streptococci are facultative anaerobes, non motile, and tend to grow in chains of variable length. Many of them are associated with serious infections in humans, such as pharyngitis, cellulitis, impetigo, lymphangitis, and acute rheumatic fever which are caused by *Streptococcus pyogenes*. The viridans group including *Streptococcus gordonii*, *Streptococcus mutans, Streptococcus salivarius*, and *Streptococcus mutor* are inhabitants of the oral cavity and commonly involved in subacute bacterial endocarditis. Dental caries is one of the most widespread bacterial infections caused by *Streptococcus mutans. Enterococcus faecalis*, a normal inhabitant of the gut, is the major pathogen in endocarditis and urinary tract infections. *Streptococcus agalactiae* is one of the common pathogens causing meningitis in newborn infants. *S. pneumoniae* is the etiologic agent of lobar pneumonia which arises in conjunction with a preexisting debilitating illness. In addition, some streptococci such as *Streptococcus lactis* and *Streptococcus cremoris* are important in the dairy industry.

Antibiotic Resistance in Pneumococci

Clinical drug resistance among bacteria is often due to R-plasmids, but also can be acquired via transposable elements or chromosomal

mutations. In streptococci two major conjugative gene transfer mechanisms have been described as being due to conjugative plasmids and conjugative transposons. Many R-plasmids are nonconjugative but can often be mobilized if they co-exist with a conjugative plasmid in a cell. Transposons are defined segments of DNA that can transpose to a different site without requiring extensive sequence homology and host recombination systems. Despite the distinct differences between transposable elements and conjugative R-plasmids of *Enterobacteriaceae* and those of gram-positive bacteria, many carry a selectable drug resistance gene.

Over the last 50 years, pneumococci acquired resistance to a number of antibiotics (70). Sulfonamide resistance appeared in clinical pneumococci in 1939 followed by tetracycline and penicillin resistance. The erythromycin resistance appeared in the 1960's. During the 1970's, chloramphenicol- and kanamycin-resistant isolates of pneumococci were reported. In 1977, dramatic appearance of multiple drug resistance in clinical pneumococci occurred worldwide (12, 23, 24,39). Most of these isolates were resistant to chloramphenicol, erythromycin or kanamycin in addition to tetracycline (39). The chloramphenicol-resistance determinant specifies a chloramphenicol acetyl transferase (product of the *cat* gene) which detoxifies chloramphenicol (47). The MLS-resistance determinant in clinical isolates of pneumococci was found to be homologous to the *erm* gene of the staphylococcal plasmid pI258 (79). The *erm* gene product modifies the 23S subunit of ribosomal RNA, resulting in resistance to macrolide, lincosomide and streptogramin B (MLS^r) antibiotics. Kanamycin resistance in pneumococci is due to aminoglycoside 3'-phosphotransferase, product of the *aphA* gene. The product of *tetM* gene, responsible for

tetracycline resistance, seems to bind to the 30S subunit of ribosome and makes it insensitive to tetracycline inhibition (29).

Even though a large number of conjugative and non-conjugative R-plasmids were identified in the genus of streptococci with the marked exception of pneumococci (8, 23, 68).

The first direct evidence for the chromosomal location of the drug-resistance determinants was provided by Shoemaker *et al.* (66). They observed that the *cat tet* determinant of the BM6001 element, and the *cat tet erm* determinants of *S. agalactiae* B109 co-sedimented with chromosomal point markers, but not with the plasmid markers, both on velocity and dye-buoyancy gradients (70). These chromosomal elements were able to transfer by a process requiring cell-to-cell contact both within and between different species of streptococci on nitrocellulose filters (69). The transfer was a DNase-resistant process and did not involve transformation or generalized transduction (31).

Conjugative Transposons of Streptococci

Over the last 15 years, a number of conjugative transposons have been characterized. These elements range in size from 15 kb to over 60 kb. The frequency of conjugative transfer of these elements during filter mating is about 10^{-8} to 10^{-4} per donor cell (8, 25, 37, 69, 70). While many carry a combination of drug-resistance determinants, most of the streptococcal conjugative transposons identified so far carry a *tetM* type tetracycline-resistance determinant (7). Among the known ones, the smallest is a 16-kb transposon, Tn*916*, conferring tetracycline resistance, isolated from *E.*

faecalis DS16 and well characterized by Clewell and coworkers (14, 15, 16, 17, 18, 19, 25, 26, 27, 28). Tn*916* is able to transpose from the chromosome onto the hemolysin plasmid, pAD1 intracellularly, and conjugate intercellularly to a number sites on the recipient chromosome. DNA sequence analysis revealed that short patches of direct and imperfect inverted repeats flanked the transposon at the termini (19). Excision of Tn*916* was seen to be precise and no target duplication was generated upon insertion. A covalently-closed monomeric circular intermediate of Tn*916* was shown to be involved in the transposition event (10, 61).

Although conjugative transposition has been observed primarily in gram-positives, Tn*916* was capable of transfer to other species including some gram-negatives either by transformation or conjugation (20). Transfer of Tn*916* among natural soil populations such as *Streptomyces* has also been documented (51).

Tn*1545*, a 25-kb conjugative transposon, conferring resistance to erythromycin, kanamycin and tetracycline was discovered in the chromosome of *S. pneumoniae* BM4200 as a part of the larger *cat tet erm aphA* element (8, 21). Tn*1545* shares substantial similarities to Tn*916* both structurally and functionally. DNA sequence analysis of its termini showed extensive homology to those of Tn*916*, suggesting common ancestry (9, 19). Tn*1545* was also capable of transfer to *E. coli* and a number of gram-positive bacteria by conjugation (22). Like *Tn916*, Tn*1545* did not generate target duplications at the site of insertion (9). The right terminus of Tn*1545* carryies two genes whose products are thought to be site-specific recombinases (*int*-Tn and *xis*-Tn). They may be involved in the excision and integration of the transposon. The amino acid sequence of these

proteins was found to be similar to those Xis and Int proteins of bacteriophage lambda suggesting possible common ancestry (53).

Tn*3701* is a composite conjugative transposon (50 kb or more), found in *S. pyogenes* A454 that encodes resistance to chloramphenicol, erythromycin and tetracycline (42). By DNA-DNA hybridization analyses, Le Bouguenec and coworkers (43) identified a 19-kb region within the Tn*3701* with significant homology to Tn*916*. This region, designated as Tn*3703*, carries both *tet* and *erm* determinants. Unlike Tn*916*, Tn*3703* seemed not to have conjugative properties.

Another composite conjugative transposon, Tn*3951*, found in *S. agalactiae* B109, is 67 kb and confers resistance to chloramphenicol, erythromycin and tetracycline (38). Smith and Guild (72) were able to mobilize Tn*3751* into *E. faecalis* by conjugation. After introducing the transposon in *E. faecalis* recipients, they introduced the hemolysin plasmid pAD1 into the same recipient. Using this strain as a donor and selecting for the transposon markers, they were able to generate a nonhemolytic derivative strain that contained Tn*3951* inserted into pAD1 which exhibited an increase in its size to about 60 kb. Tn*3951* was further characterized and mapped (38, 42). The *tet* and *erm* determinants were cloned on a single 22-kb *Eco*RI fragment. Both tetracycline resistant (Tc^r) and erythromycin resistant (Em^r) determinants were found to be capable of expressing in the *E. coli* DB11 strain. The *cat* determinant which resides on a 6.6-kb *Eco*RI fragment was found to be noncontiguous with the *tet-erm* fragment. Like Tn*3701*, the *erm* and *tet* determinants of Tn*3951* showed extensive homology to Tn*916*. Insertion of Tn*3951* in the pneumococcal chromosome following conjugal transfer was found to be site-specific (57).

Two Tn*916*-like transposons, Tn*5381* and Tn*5383*, carrying tetracycline resistance (*tetM*) have been identified recently in clinical isolates of *E. faecalis* (57). Of the two, Tn*5381* was shown to form a closed-circular intermediate following excision from the host chromosome. Increase in the conjugal transfer frequency and circular- intermediate formation were shown to be increased 10-fold in the presence of a subinhibitory concentration of tetracycline.

Studies in this work have focused on the Ω(*cat tet*) element of *S. pneumoniae* . Transfer frequency of the *cat* and *tet* determinants to other streptococci during the filter matings was 10^{-7} to 10^{-6} per donor cell (70). Based on transformation experiments, the *cat* and *tet* determinants were found to be linked together, and they co-migrated with the chromosomal markers in velocity sedimentation and buoyancy gradients, eliminating the possibility of any plasmid forms. By inserting an *E. coli* vector plasmid, pVA891, at a number of sites specifically within the transposon Vijayakumar and coworkers (76) were able to clone and to recover segments of the element in *E. coli*. The physical analysis of the passenger DNA fragments from these plasmids enabled them to construct a detailed restriction map of this element (77). Ayoubi *et al.* (3) have recently shown that the internal Tn*916*-like region (Tn*5251*) is capable of independent conjugative transposition. The remaining 47.5-kb element carrying the *cat* determinant (Tn*5252*) is still capable of conjugative transposition. Tn*5252*, unlike Tn*5251*, preferred to insert at a unique site on the recipient chromosome by a site-specific integration mechanism (78), similar to the behavior of the parental transposon, Tn*5253*. Tn*5251*, therefore, did not play a mechanistic role in the conjugal transfer of the parental transposon. These data strongly suggested that the larger conjugative transposons such as

Tn*5253*, Tn*3951*, and Tn*3701* were made of smaller discrete elements which may be capable of independent movement when removed from the context of the parental element. The Tn*916*-like element from within the larger transposon Tn*5253*, also suggested the existence of two different classes of transposons whose genetic organizations and functional features could be distinct from those of the Tn*916*-class of elements.

The presence of a fully functional transposon and two IS-like elements within the larger transposon indicate that the evolutionary origin and genetic organization of Tn*5252* may be somewhat complicated. The two direct repeats flanking the *cat* gene in Tn*5252* were found to be similar to the staphylococcal plasmid, pC194 (70). In addition, some portion of the DNA at the right end of Tn*5252* has been shown to be homologous to the Rx1 chromosome (77). Further work may provide more information on the autoaccumulation of various genes into prototype conjugative transposons such as Tn*5252* and Tn*3701* which might have given give rise to the larger composite elements like Tn*5253*.

Genetic Analysis of Conjugative Transposons

By using Tn*5* as a tool, numerous insertions have been introduced within Tn*916* carried on *E. coli* vector plasmid pVA891 in *E. coli* to study the transfer-related genes in this element (63, 82). After the insertions of Tn*5* within the transposon were determined by restriction mapping, they were introduced into *E. faecalis* via protoplast transformation. The Tc^r transformants devoid of erythromycin represented Tn*916* insertions in the host chromosome. Tn*5* insertions were generated over the length of Tn*916*,

and most of these mutants were able to insert into the *E. faecalis* chromosome. The subsequent Tn*916* transformants of *E. faecalis* cells were used as donors in filter matings to test the conjugal-transfer ability of Tn*916*. Intracellular-transposition ability of Tn*916* from *E. faecalis* chromosome to plasmid pAD1 was also determined. Tn*5* insertions within a 1-kb region near the left end of Tn*916* were found to be essential for its excision and establishment in *E. faecalis*. These mutants were also unable to undergo excision in the absence of tetracycline in *E. coli*. However, excision occurred readily when the wild-type region was provided *in trans* on a different plasmid (63). The majority of Tn*916*::Tn*5* insertions that were able to transform *E. faecalis* were unable to undergo conjugal transfer, and some of these were incapable of intracellular transposition to plasmid pAD1. Insertions close to the *tet* gene and two insertions close to the right end did not have any effect on the transfer of the transposon.

Poyart-Salmeron and coworkers (53) determined the genetic organization of Tn*1545* by introducing a series of large deletions within the internal portion of the transposon that maintained the kanamycin determinant and at least 185 bp at each end. They identified a region of about 2 kb to the right end of the transposon (equivalent to the left end of Tn*916*) that was essential for the excision of the transposon from the plasmid and subsequent transposition. Excision of this deletion derivative, Tn*1545*-Δ4, could be complemented *in vivo* by providing the appropriate segment of DNA on a plasmid vector. Sequence analyses of this region led to the identification of two proteins, designated Xis-Tn and Int-Tn that are involved in excision of the element.

These two proteins were found to be essentially identical to those of Tn*916* (74) which were essential in the excision of Tn*916* in *E. coli*. These

products exhibited homology with the excisinase (Xis) and integrases (Int) of lambdoid bacteriophages suggesting that they evolved from a common ancestor (54).

The Xis-Tn and Int-Tn are basic proteins (75) and exhibit similarity to the Xis protein of bacteriophage P22 and to the family of Int-related site-specific recombinases, respectively (53). These recombinases include phage-encoded integrases, transposon-encoded transposases, resolvases and invertases. The carboxyl termini of Int-family proteins of these transposons and bacteriophages are highly conserved and believed to be part of their active sites (2, 44).

The goal of this study was to identify and localize the DNA sequences involved in the transfer of Tn*5252*. Among the known composite conjugative transposons, Tn*5252* was the first one identified as an independent transposon. There has been no information on the genetic organization of Tn*5252*-like transposons to date. Because of its large size, it was not possible to clone the entire Tn*5252* or mobilize it by transposition on a plasmid vector. Hence, I was unable to perform Tn*5* mutagenesis as done with Tn*916* or introduce deletions as describe for Tn*1545*. Instead, introducing a number of insertions and deletions within Tn*5252* was possible by using an alternate technique. However, this method had limitations and was heavily dependent upon the presence of appropriate restriction sites within the transposon. This approach, however, enabled me to identify specific regions that are essential for the transfer of the element. Subsequently, localization of these regions facilitated the characterization of some of them at the molecular level to provide insight into their role in the conjugative transposition of Tn*5252*.

CHAPTER III

MATERIAL AND METHODS

Bacterial Strains and Plasmids

All *S. pneumoniae* strains used in this study were derivatives of the non-encapsulated "wild-type" laboratory strain of Rx1. Rx1 is a subclone of Rx, derived from Avery's R36A strain (65). DP1322 is Rx1 carrying Tn*5253*, and DP1324 is DP1322 carrying the *str-1* point mutation conferring resistance to streptomycin (Str^r). DP1333 is DP1322 which acquired *tet-3* point mutation and spontaneously lost the *cat* determinant and is sensitive to tetracycline (Tc^s) and chloramphenicol (Cm^s) respectively. DP1617 has multiple point mutations, and its DNA is used as a source of chromosomal markers and reference donor DNA in transformation. Bacterial strains used in this work are listed in Table I. *S. pyogenes* ATCC 21547 was obtained from Stillwater Medical Center. *S. gordonii* (formally called *S. sanguis* Challis), and the recombination deficient strain of *E. faecalis* UV202 (81) with *rif* and *fus* chromosomal markers conferring resistance to rifampicin (Rif^r) and fusidic acid (Fus^r) respectively were used in conjugation experiments. *Bacillus subtilis* JH642 strains, kindly provided by P. Zuber, were also used as recipients in some of the conjugation experiments.

TABLE I

BACTERIAL STRAINS

Strain	Genotype	Origin/Reference
S. pneumoniae		
Rx1	*hex*	(65)
DP1002	*nov-1*	(30)
DP1004	*str-1*	(30)
DP1322	Tn*5253* (*cat tet*)	(70)
DP1324	*str-1* Tn*5253* (*cat tet*)	(67)
DP1333	Tn*5253* (Δ*cat tet-3*)	(70)
DP1617	*hex str-1 ery-2 nov-1 fus sulf-d stg*	(70)
DP1355	*nov-1* Tn*916* (*tet*)	(55)
DP3220	*str-1* pIP501 (Cm Em)	(30)
GP42	*str-1* Tn*5253* (*cat* ΩEm Tc^s)	(76)
SP1000	*str-1 fus* Tn*5252* (*cat*)	(3)
SP1200	*str-1 fus* Tn*5252* (*cat* ΩEm) Tra -	(1)
SP1201	*str-1 fus* Tn*5252* (*cat* ΩEm) Tra -	(1)
SP1202	*str-1 fus* Tn*5252* (*cat* ΩEm) Tra -	This study
SP1203	*str-1 fus* Tn*5252* (*cat* ΩEm) Tra -	This study
SP1204	*str-1 fus* Tn*5252* (*cat* ΩEm) Tra -	This study
SP1205	*str-1 fus* Tn*5252* (*cat* ΩEm) Tra -	This study
SP1252	*str-1 fus* Tn*5252* (*cat* ΩEm) Tra -	(1)
SP1253	*str-1 fus* Tn*5252* (*cat* ΩEm) Tra -	This study
SP1254	*str-1 fus* Tn*5252* (*cat* ΩEm) Tra +	This study
SP1255	*str-1 fus* Tn*5252* (*cat* ΩEm) Tra +	This study
SP1256	*str-1 fus* Tn*5252* (*cat* ΩEm) Tra -	This study
SP1257	*str-1 fus* Tn*5252* (*cat* ΩEm) Tra +	This study

TABLE I contd.

SP1258	*str-1 fus* Tn*5252* (*cat* ΩEm) Tra +	This study
SP1259	*str-1 fus* Tn*5252* (*cat* ΩEm) Tra -	This study
SP1260	*str-1 fus* Tn*5252* (*cat* ΩEm) Tra +	This study
SP1704	*nov-1* Tn*5251* (*tet*)	This study
SP1705	*nov-1* Tn*5251* (*tet*)	This study
SP1706	*nov-1* Tn*5251* (*tet*)	This study
Enterococcus faecalis		
UV202	*fus rif*	(81)
Streptococcus pyogenes		
ATCC 21547	*opt*	Stillwater Med. Cent.
SY152	*opt* Tn*5252* (*cat* ΩEm) Tra +	This study
SY153	*opt* Tn*5252* (*cat* ΩEm) Tra +	This study
SY154	*opt* Tn*5252* (*cat* ΩEm) Tra +	This study
SY158	*opt* Tn*5252* (*cat* ΩEm) Tra +	This study
SY159	*opt* Tn*5252* (*cat* ΩEm) Tra +	This study
Bacillus subtilis		
JH642	*sigB trpC2 pheA sigB::cat*	P. Zuber
JH642	*spoVG lacZ trpC2 pheA spoVG*::pZL207 (Sp^r)	P. Zuber

Recombination deficient *E. coli* strains (Table II) were used for generation and purification of recombinant plasmids. The recombination proficient *E. coli* C600 strain was used as a recipient in some of the conjugation experiments with donors carrying Tn*5251*.

The vector plasmid, pVA891, can replicate autonomously and confers resistance to erythromycin and chloramphenicol in *E. coli*. It expresses only erythromycin resistance when it is inserted into the streptococcal chromosome. Also it is incapable of autonomous replication in the streptococci. Other cloning vectors used were mainly the pUC series or pUC derivatives (Table II).

Enzymes, Chemicals, and Reagents

Restriction endonucleases, T4 DNA ligase, *E. coli* DNA polymerase I, DNA molecular weight standards and DNA Sequencing Kit were purchased from Promega Biotec, Bethesda Research Laboratories (BRL), United States Biochemical Company (USBC), International Biotechnologies Inc. (IBI) or New England Biolabs (NEB). Antibiotics, DNase I, RNaseI, egg white lysozyme (grade 1), and bovine serum albumin (fraction V) were purchased from Sigma. Bacteriological agar and media were purchased from Difco. X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactoside) and IPTG (isopropyl-β-D thiogalactopyranoside) were purchased from Bethesda Research Laboratories. Agarose and polyacrylamide from Fisher, and ultrapure

TABLE II

E. coli STRAINS AND CLONING VECTORS

Strain	Genotype/Phenotype	Source/Reference
DH1	*supE*44 *hsdR*17 *endA*1 *gyrA*96 *thi*-1 *recA*1 *relA*1	Bethesda Research Laboratories
DH5α	*supE*44 Δ*lac*Y169 (Φ80*lacZ*ΔM15) *hsdR*17 *recA*1 *endA*1 *gyrA*96 *thi*-1 *relA*1	Bethesda Research Laboratories
C600	*supE*44 *hsdR thi*-1 *thr*-1 *leuB6 lacY*1 *tonA*21	
JM109	*recA*1 *supE*44*endA*1 *hsdR*17 *gyrA*96 *relA*1 *thi*Δ (*lac-proAB*) F' [*traD*36 *proAB* +*lacI* ᑫ*lacZ*ΔM15]	
pUC8	2.7 kb, *lacZ*, Ap^r	
pBluescript SK+	2.9 kb, *lacZ*, Ap^r	Stratagene
pVA891	5.9 kb, Cm^r, Em^r	(45)

Cm: chloramphenicol, Em: erythromycin, Ap: ampicillin

agarose from Bio-Rad Laboratories were used for gel electrophoresis and DNA sequencing. [α-^{32} P] dCTP and [α-^{35} S] dATP were purchased from New England Nuclear (NEN). Other chemicals, compounds and reagents were purchased from either Sigma, Fisher or USBC.

Media, Growth Conditions, and Storage of Bacteria

S. pneumoniae, S. pyogenes, S. gordonii and *E. faecalis* were grown and stored in a complex broth medium (CAT) without aeration. CAT is a rich medium containing 10 g of casein hydrolysate, 5 g of tryptone, 1 g of yeast extract, and 5 g of NaCl per liter of deionized water. For solid CAT medium, 1.5% (w/v) agar was added to CAT broth prior to autoclaving. After autoclaving CAT agar and CAT broth were cooled to 60°C and to room temperature respectively and aseptically supplemented with 0.016 M K_2HPO_4 and 0.5% (w/v) glucose before use. Since pneumococci tend to autolyse if grown to high densities, they were routinely grown up to an $OD_{550\ nm}$ of 0.2-0.4 for immediate use or storage in 10% (v/v) glycerol at -80°C. For pneumococcal strains an $OD_{550\ nm}$ of 0.2 was found to give approximately $2x10^8$ CFU/ml. To provide an anaerobic environment, pneumococcal strains were grown embedded between CAT agar layers supplemented with 5% sheep or bovine blood. Other streptococcal strains were routinely grown without aeration for a minimum of 16 h in CAT broth and stored as described for the pneumococci. On the other hand, *E. faecalis*, *S. pyogenes*, and *S. gordonii* were grown on CAT agar surfaces unless they were mated with pneumococci at which time they were embedded between

layers of CAT agar. Selective drug concentrations for bacterial strains are given in Table III.

Preparation of Competence Cells and Transformation

Preparation of Pneumococcal Competent Cells

Pneumococcal competent cells were prepared by the methods described by Guild and Shoemaker (65) and stored in 10% glycerol at -80°C. Cells were grown in mid-exponential phase in CAT medium (about $2x10^8$ CFU/ml, an $OD_{550\ nm}$ of 0.2), and then diluted 100 fold into competence medium (CTM), and incubated at 37°C. CTM is prepared the same as CAT broth except it is supplemented with 10 mM $CaCl_2$ and 0.2% (w/v) bovine serum albumin (Fraction V). Starting at 70 min, 1 ml of culture was added to tubes containing 0.1 ml glycerol, mixed and frozen in acetone-dry ice for 5 min and stored at -80°C. Samples were taken at 10 min intervals for up to 140 min. The stored cells were then thawed and tested for competence. The time of peak competence was calculated by plotting the number of transformants as a function of time on graph paper. Once optimum competence time was calculated, a 250-ml culture from the same batch of medium was inoculated and aliquoted to 25 ml tubes (20x150 mm) containing 2.5 ml glycerol, and quickly frozen. Because the half width of the sharp competence peak is often around 10 min or less, samples were taken 10 min earlier than the sharp competence peak time.

TABLE III

SELECTIVE ANTIBIOTIC CONCENTRATIONS

Genotype/ Phenotype	Antibiotic	Concentration (μg/ml) stab plate/broth	overlay
Streptococcus pneumoniae			
cat (Tn*5253*)	chloramphenicol	5	15
Em^r	erythromycin	3	5
fus	fusidic acid	10	50
nov	novobiocin	10	10
rif	rifampicin	10	10
str	streptomycin	200	600
tet	tetracycline	2	5
spc	spectinomycin	200	500
Enterococcus faecalis			
cat (Tn*5253*)	chloramphenicol	25	75
fus	fusidic acid	25	50
rif	rifampicin	25	50
str	streptomycin	200	1000
et	tetracycline	4	12
Streptococcus pyogenes			
cat (Tn*5253*)	chloramphenicol	5	10
Em^r	erythromycin	1	5
nov	novobiocin	20	20
str	streptomycin	600	1000
tet	tetracycline	2	5
opt	optochin	20	50

TABLE III contd.

Bacillus subtilis

Cm^r	chloramphenicol	10
tet	tetracycline	10
Em^r	erythromycin	1
nov	novobiocin	10
str	streptomycin	200
opt	optochin	20

Escherichia coli

Cm^r	chloramphenicol	10
Tc^r	tetracycline	10
Em^r	erythromycin	200
Km^r	kanamycin	50
Ap^r	ampicillin	50
Sm^r	spectinomycin	200

Transformation of Pneumococcus

Frozen competent recipient cells were thawed on ice, mixed with donor DNA, and incubated for 30 min at 37°C. Pancreatic DNase I solution in CAT broth was added at a final concentration of 10 μg/ml to the cultures, and the cultures were kept at 37°C for 5 min before plating. Appropriate dilutions of the cultures were made in 5 ml CAT broth with 3% blood and mixed with 5 ml CAT agar cooled to 49°C, and poured on plates containing 20 ml base agar. After the cell layer solidified, the plates were overlaid with 10 ml of agar and incubated for 90 min at 37 °C to allow phenotypic expression. The plates were overlaid with 10 ml of selective agar and incubated at 37°C for 18 to 24 h.

Transformation of *E. coli*

Competence regimen and transformation of *E. coli* with plasmid DNA were done by the methods described by Hanahan (32). The competent cells were frozen and stored at -80°C for up to two months. Transformed cells were diluted and plated immediately on selective LB plates. Occasionally, in the case of ampicillin selection, transformed cells were subcultured in the presence of ampicillin for 12 h to reduce the appearance of satellite colonies before plating. For phenotypic differentiation of Lac^- transformants, IPTG (5 mM) and X-gal (40 μg/ml) were added to the selective plates.

Conjugation

Conjugation between pneumococcal donors and recipients was performed as described by Smith and Guild (69) with some modifications. Both donor and recipient cells were grown in CAT broth and mixed at a ratio of 1:5 (donor:recipient) in the presence of 10 mM $MgSO_4$, 2 mg/ml of bovine serum albumin (BSA), and 100 μg/ml DNase I. The mixture of cells containing about $6x10^8$ cells was filtered through nitrocellulose filters (Millipore: with 13 mm diameter and 45 μm pore size). The filter was placed, cells down, on CAT agar containing 10 mM $MgSO_4$, 2 mg/ml BSA and 70 μg/ml DNase I, and then overlaid with the same agar. After 4 h of incubation at 37°C, the filter with the agar bottom and top were removed and washed in 3 ml CAT broth with 10 mM $MgSO_4$, 2 mg/ml BSA, 10 μg/ml DNase I, and 10% glycerol by vortexing. The liquid from the filter wash was decanted, diluted, plated, and overlaid with selective agar to score for the transconjugants as well as the viability of parental strains. The remainder of the culture was stored at -80°C for future plating.

Conjugation between pneumococcal strains and *E. faecalis, S. pyogenes, S. gordonii, B. subtilis*, and *E. coli* was performed under the same conditions except that the conjugation time varied from 4 h to 22 h, and CAT medium was supplemented with 1 mM $CaCl_2$. All parental cells, except pneumococci were plated directly on the surface of selective agar plates to score for the transconjugant and the viability of the parental strains. The use of Difco Bacto casitone rather than USBC casein hydrolysate in CAT medium was found to increase the conjugation frequency among pneumococcal and other streptococcal strains (3).

Drug resistance for pneumococcal strains was scored by plating the cells on CAT-base agar which was overlaid with two layers of agar. For single and double selection, drugs were added to the top agar layer to select for both chromosomal (three-letter phenotypes) and transposon or plasmid markers (two-letter phenotypes). The agar layer which separates the cell layer and drug layer, serves as a buffer layer, and enables the cells to be induced with a low level of drug before the selective concentration of drug arrives at the cell layer . Although induction was not required for chromosomal point mutations, all but streptomycin (Str) was applied to the drug layer because it was found that addition of streptomycin to all three agar layers (200 μg/ml) greatly reduced the background colonies and facilitated the observation of transconjugants.

The transconjugants were individually checked for unselected markers by replica plating onto selective CAT agar containing 2% blood.

Chromosomal and Plasmid DNA Isolation

Chromosomal DNA Isolation from Pneumococcus

DNA from pneumococci was purified according to the method described by Marmur (46). Cells were cultured in broth without aeration to a density of about 3 x10^8 cells/ml. After adding 10 mM of EDTA to the culture, it was swirled and kept on ice for 10 min. The culture was centrifuged at 4°C for 10 min at 5,800 xg in a Sorvall GSA rotor in a Sorvall RC-5B Centrifuge (DuPont Instruments). The pellet was resuspended in 25

ml SSC (150 mM NaCl, 15 mM sodium citrate). The resuspension was transferred to 50 ml polypropylene Oak Ridge tubes and centrifuged at 4°C for 10 min at 9,200 xg in a Sorvall SA-600 rotor. The pellet was resuspended in 3 ml of lysis solution [30 mM EDTA, 0.1% (w/v) sodium dodecyl sulfate (SDS), 0.1% sodium deoxycholate (DOC), 0.4% (v/v) triton X-100, 100 μg/ml RNase, and 0.1% sarkosyl] and incubated at 37°C for 10 min or until the solution cleared indicating lysis of the cells. After lysis, 0.1% (v/v) diethyl pyrocarbonate (Sigma) was added and the mixture was held at 65°C for another 30 min to inactivate nucleases. The viscous and colorless lysate was then extracted with an equal volume of phenol-chloroform-isoamyl alcohol (25: 24:1), twice with chloroform-isoamyl alcohol (24:1), and precipitated with two volumes of 100% ethanol at -20°C for 30 min. The DNA was pelleted by centrifugation at 4°C at 20,800 xg for 10 min. The pellet was vacuum dried (Forma-Vac, Forma Scientific, Inc.) at 65°C and resuspended in TE buffer. Concentration of the DNA was measured in a UV spectrophotometer (Spectronic 1001, Milton Roy Co.) and stored at -20°C.

Chromosomal DNA Isolation from *Streptococcus pyogenes*

Chromosomal DNA extraction from *S. pyogenes* was performed essentially by the methods described by Clewell and Franke (13) with some modifications. The CAT broth medium was used as a growth medium. Cells were grown in a 250-ml CAT broth at an $OD_{550\ nm}$ of 0.4 to 0.5. The cells were pelleted and washed in 20 ml of TES buffer [0.03 M Tris-HCl, 0.005 M EDTA and 0.05 M NaCl (pH 8.0)]. The pellet was resuspended in

5 ml of a solution containing 25% of glucose, 0.03 M EDTA and 5 mg of lysozyme in TES buffer. The suspension was allowed to incubate for 1 h at 37°C. The cells were then lysed by addition of 2 ml of 2% SDS and 0.1% of DOC followed by phenol-chloroform extraction and ethanol precipitation as described for pneumococci. This DNA was used in blot hybridization or as donor DNA in transformation experiments.

Plasmid DNA Isolation from *E. coli*

Alkaline-SDS lysis and rapid plasmid DNA isolation from *E. coli* were essentially done according to Sambrook *et al.*(59). One and a half ml of freshly grown cells was centrifuged in a microfuge tube at 11,750 xg at room temperature for 30 sec in a microcentrifuge (Eppendorf Centrifuge 5415 C). Cells were resuspended in 100 µl of cell resuspension solution [50 mM glucose, 25 mM Tris-HCl (pH 8.0), and 10 mM EDTA (pH 8.0)] by vigorously vortexing, and kept at room temperature for 5 min. Cells were lysed with 200 µl of freshly prepared lysis solution (0.2 N NaOH, 1% SDS) on ice for 5 min. The lysate was mixed with 150 µl ice cold neutralization solution [3 M potassium acetate (pH 4.9) and 11.2% (v/v) glacial acetic acid], and held on ice for a further 5 min. The lysate was centrifuged at 11,750 xg at room temperature twice for 5 min, and the supernatant was transferred to a fresh tube. Plasmid DNA was precipitated with two volumes of 95% ethanol at -20°C for 15 min. The DNA was pelleted at 11,750 xg for 5 min, vacuum dried, and resuspended in 100 µl TE buffer containing DNase-free pancreatic RNase (20 µg/ml). A 5 µl of DNA sample was electrophoresed on an agarose gel for rapid screening of the recombinant

plasmids. For restriction endonuclease analysis, the plasmid DNA was further cleaned with equal volumes of phenol-chloroform, and chloroform-isoamyl alcohol, before ethanol precipitation, and resuspended in 50 μl of TE buffer.

Large scale plasmid DNA purification was essentially done the same as the mini-plasmid DNA purification protocol except for the scale-up preparation and the use of CsCl. A 250 ml culture of *E. coli* was grown with vigorous shaking at 37°C for 12 h and used directly for multicopy plasmid DNA purification. An overnight culture of *E. coli* harboring a low copy number plasmid was supplemented with spectinomycin (300 μg/ml) for plasmid amplification and incubated for an additional 12 h. The culture was then centrifuged at 5,800 xg for 10 min at 4°C. The cells were resuspended in 5 ml of resuspension solution and transferred to an Oak Ridge tube. Ten mililiters of lysis solution was then added to the mixture and held on ice for 10 min followed by addition of 5 ml of neutralization solution on ice for a further 30 min. The lysate was centrifuged at 20,800 xg for 20 min at 4°C, and plasmid DNA was precipitated with equal volume of isopropanol at room temperature for 30 min. The DNA was pelleted at 20,800 xg at 17°C for 20 min. The pellet was washed with 1 ml of 70% ethanol, vacuum dried and resuspended in 4 ml of TE buffer. The DNA solution was mixed with 1 g/ml (w/v) CsCl and 1 mg/ml (w/v) ethidium bromide, and centrifuged at 227,640 xg in a VTi65 rotor at 17°C for 18 h (or 383,700 xg for 4 h) in the XL-70 ultracentrifuge (Beckman). The plasmid DNA band was removed using a 16-G needle and a 3-ml plastic syringe, extracted with water saturated butanol several times, and dialyzed against 2 liters of TE buffer for 18 h.

DNA Manipulation and Analysis

Conditions employed for digestion of DNA with restriction endonucleases, cloning, alkaline phosphatase treatment of vector DNA were essentially as recommended by the manufacturers, and according to Sambrook *et al.* (59). Subcloning of pneumococcal DNA fragments in *E. coli* was performed in a 20 µl volume reaction mixture by using less than 5 µg DNA at a ratio of one vector to five insert at 16°C for 24 h. For deletion and insertion mutagenesis, the insert:vector ratio was 1:1 in a 60 µl reaction mixture with 5 µg of DNA. In the latter case the ligation mixture was extracted with phenol-chloroform, linearized with a restriction endonuclease and then used as donor DNA to transform competent SP1000 cells.

Agarose Gel Electrophoresis and Restriction Endonuclease Mapping of Recombinant DNA

Agarose gel electrophoresis of plasmid and chromosomal DNAs was performed as described by Sambrook *et al.* (59). For blot hybridization of chromosomal DNA, and measurement of DNA fragments larger than 2.0 kb, 0.6 to 0.8% agarose gels were used. For measurements of fragments smaller than 2 kb, 1.2% agarose gels were used. DNA samples were mixed with a tracking solution containing 5% glycerol, 3 mM EDTA (pH 8.0), bromophenol blue, and xylene cyanol. Electrophoresis was done in TBE buffer [89 mM Tris base, 89 mM boric acid and 2.5 mM EDTA (pH 8.0)] at room temperature. Gels were stained for 15 min in 1 µg ethidium bromide per ml of water, destained in deionized water for 30 min, transilluminated

with 300 nm ultraviolet light, and photographed with a Kodak polaroid camera. Fragment sizes were estimated by comparison to molecular weight standards (*Hin*dIII fragments of phage lambda and *Hae*III fragments of phage ϕX174).

To purify specific DNA restriction fragments, 0.6 to 1.0% preparative mini-gels made with Ultra Pure agarose (Bio-Rad) were used. After destaining, a gel slice containing the DNA fragment was electroeluted into a dialysis bag and purified according to Sambrook *et al.* (59).

Southern Hybridization

Preparation of Membrane-bound Denatured DNA

The blot hybridization was carried out essentially by the methods described by Southern (73), Sambrook *et al.* (59), and according to manufacturer's recommendations. After electrophoresis, the gel was soaked in 200 ml of 0.5 N NaOH for 30 min to denature the DNA. A pre-cut nylon membrane (GeneScreen Plus, NEN), and a sheet of filter paper the same size as the nylon membrane were wetted in deionized water for 10 min and placed on a vacuum blotter (Model 785 Vacuum Blotter, Bio-Rad). The DNA was then transferred to the nylon membrane using 10x SSC transfer solution (3.0 M NaCl and 0.3 M sodium citrate) for 90 min at 5 Hg/inch. After transfer, the membrane was briefly rinsed in 2x SSC and air dried between two pieces of filter paper.

Prehybridization and Hybridization

The DNA-bound membrane was placed into a heat sealable plastic bag containing 10 ml of prehybridization solution (1 M NaCl, 1% SDS, and 10% dextran sulfate). The bag was incubated at 65°C for more than 6 h by slow agitation. One corner of the bag was cut and the heat denatured probe was added to 2 to $5x10^6$ cpm per bag. Hybridization was carried out at 65°C for 12 h with agitation. The membrane was removed from the bag and washed under high stringency conditions as recommended by the manufacturer. The membrane was washed twice in 2x SSPE buffer [17.53% (w/v) of NaCl, 2.76% (w/v) of NaH_2PO4, and 0.74% (w/v) of EDTA, pH 7.4] for 15 min at room temperature, twice in 2x SSPE, 2% SDS for 45 min at 65°C, twice in 0.1x SSPE buffer for 30 min at room temperature, and once in 3 mM Tris base (unbuffered) solution for 15 min at room temperature. The membrane was briefly air dried, wrapped in a plastic wrap and exposed to X-ray film (Kodak X-OMAT AR) with an intensifying screen at -80°C for varying times.

Nick Translation

Nick translation of probe DNA with [α-^{32}P] dCTP was performed according to standard methods described by Sambrook *et al.* (59). The reaction mixture contained 0.5 to1.0 µg of probe DNA, 5 µl of 10x buffered nucleotide mix (10 mM of dGTP, dATP, and dTTP in 50 mM Tris-HCl, pH 7.5 and 10 mM $MgSO_4$), 0.5 µl DNase I (1 U/µl), 5 µl of [α-^{32}P] dCTP

(10 mCi/ml), 0.5 μl DNA polymerase I (9000 U/μl), and sterile water to attain a final volume of 50 μl . One microliter of a 1:50 dilution was transferred in 5 ml of bioflour cocktail (Beckman) to count the total radioactivity in a Beckman LS6000C model scintillation counter. The reaction mixture was incubated at 16°C for 2 h and then stopped by adding 25 μl of 0.5 M EDTA (pH 8.0). The labeled probe was purified by ethanol precipitation after addition of 25 μl of salmon sperm DNA (2 mg/ml), 25 μl of 7.5 M NH_4 acetate, and 50 μl of TE buffer at -20°C for 20 min. The DNA pellet was washed with 70% ethanol and dissolved in 0.6 ml of TE buffer. Incorporation of radioactive phosphorus to the probe DNA was measured as described previously, and the total volume of the probe was brought to 2 ml in a test tube by adding 0.1 ml of 20x SSC, 0.4 ml of salmon sperm DNA, and 0.9 ml of sterile deionized water.

The radioactively labeled probe was denatured at 95°C for 10 min, cooled to 0°C, and used immediately or kept at -20°C until needed.

DNA Sequencing

Dideoxy chain termination DNA sequencing was carried out with Sequenase Version 2.0 kit purchased from USBC and deoxyadenosine 5'-[α-thio] triphosphate, [^{35}S]-(12.5 mCi/ml) was purchased from NEN.

Preparation of Polyacrylamide Sequencing Gel

The 7% polyacrylamide gel contained a concentration gradient in the standard TBE buffer [0.89 mM Tris-base (pH 8.3), 0.89 mM boric acid, and 2 mM Na_2EDTA] and was prepared according to the methods described by Biggin *et al.* (4). The two solutions used to make the buffer gradient gel were prepared as follows: Solution I contained 17.5% (v/v) of 40% polyacrylamide solution (38% acrylamide, and 2% bis-acrylamide), 25% (w/v) of urea, and 10% (v/v) 10X TBE buffer, solution II was made in solution I except it was supplemented with 25% of (w/v) sucrose and 0.005 gm (w/v) of bromophenol blue. Immediately before poring the gel, 0.3% (w/v) ammonium persulfate and 0.05% of TEMED (N, N, N', N'-tetramethylethylanediamine) were added to both solutions. A gel apparatus (Sequi-Gen Nucleic Acid Sequencing Cell, Bio-Rad) was filled with the denser solution (solution II) at the bottom of the gel at a ratio of approximately 1:5 (solution II:solution I) and then the chamber was filled to the top with solution I. The gel was allowed to solidify at room temperature for 18 h.

Preparation of double-stranded- and single-stranded-DNA Templates

Double-stranded DNA (dsDNA) templates were extracted with a Magic Miniprep kit (Promega) as described by the supplier except that 3 fold more cells were used. The cells were pelleted in a microfuge tube and the pellet was resuspended in 200 µl of cell resuspension solution [50 mM Tris (pH 7.5), 10 mM EDTA, and 100 µg/ml RNase A). Cells were lysed by

adding 200 µl of lysis solution (0.2 M NaOH and 1% SDS) at room temperature. The denatured lysate was then neutralized with 200 µl of solution (2.55 M potassium acetate), mixed well and kept at room temperature 5 min. The mixture was centrifuged twice at 11,750 xg for 5 min. The supernatant was transferred to a fresh eppendorf tube. Two volumes of DNA purification resin were mixed with the lysate and the DNA separated in the purification column. The column was washed with a wash solution [0.2 M NaCl, 20 mM Tris (pH 7.5), 5 mM EDTA, and 50% ethanol] and excess amount of the wash solution was removed from the column by brief centrifugation. The DNA was removed from the column with 100 µl of preheated (65°C) TE buffer and stored at -20°C.

Single-stranded DNA (ssDNA) templates were essentially prepared as described by Sambrook *et al.* (59) and according to the manufacturer's recommendations. DNA fragments cloned on pBluescript SK+ vector (Stratagene) in *E. coli* JM109 strain was cultured in 5 ml 2x YT-broth (10 g tryptone, 10 g yeast extract, and 5 g NaCl per liter) with ampicillin (50 µg/ml) for 12 h at 37°C. Fifty milliliter of 2x YT-broth containing 0.001% thiamine and ampicillin was inoculated with 1 ml of overnight starter culture and incubated at 37°C for 30 min. The M13K07 helper phage was then added (approximately 10^{10} PFU/ml) to the culture and incubated at 37°C for one h. The culture was mixed with 50 ml fresh YT broth containing kanamycin and ampicillin at a final concentration of 70 µg/ml and 50 µg/ml respectively and incubated at 37°C overnight. The next day, the culture was centrifuged twice at 9,200 xg in a Sorvall SA-600 rotor for 30 min to pellet the bacterial cells. The supernatant containing the f1 phage particles was mixed with 5% (w/v) polyethylene glycol (PEG) and 0.8 M ammonium acetate overnight on ice. The phage particles were pelleted by

centrifugation at 9,200 xg for 45 min and resuspended in 0.5 ml of TE buffer and transferred to a microfuge tube. Following several phenol-chloroform-isoamyl alcohol and chloroform-isoamyl alcohol extractions, the ssDNA was precipitated with ethanol, washed with 70% ethanol, and vacuum dried at 65°C. The DNA was resuspended in 100 µl of TE buffer and stored at -20°C. A 5 to 7 µl of this preparation was used for sequencing.

Construction of Nested Deletions

Exonuclease III, a 3' to 5' double-strand-specific exonuclease, digestion was performed on fragments over 1.0 kb in size to be sequenced on the pBluescript SK+ vector. The recombinant plasmid was digested with two restriction enzymes between the passenger fragment and the multiple cloning site of the vector. The restriction enzymes were selected so that they left a 3'-protruding end, which is not a suitable substrate for exonuclease III, near the primer binding site, and a 5'-protruding end near the end of the insert fragment. Digestion of the passenger DNA with exonuclease III from one strand (5' protruding end) was allowed to progress for an appropriate time. The single-strand ends were then treated with S1 nuclease to generate blunt ends. These ends were then ligated to circularize the plasmids. The nested deletion assay was performed as follows: A volume of 90 µl (approximately 5 µg of DNA) of DNA digested with the two enzymes was mixed with 10 µl of 10x exonuclease III buffer [0.6 M Tris-HCl (pH 8.0), 6 mM $MgCl_2$] and exonuclease III enzyme (450 units per assay) on ice. The reaction mixture was transferred to room temperature and 10 µl aliquots were removed every min for 10 consecutive min. These samples were then

transferred to 10 eppendorf tubes, each containing 30 μl of S1 nuclease solution which consisted of 30 units of S1 nuclease in 1% glycerol, 60 mM NaCl, 1.3 mM $ZnSO_4$, 8 mM potassium acetate (pH 4.6) on ice. The reaction was allow to continue at room temperature for 20 min, and was terminated with 4 μl of S1 stop buffer [50 mM EDTA and 0.7 M Tris-base (pH 8.0)]. Two samples were made by combining tubes 1 through 5 and 6 through 10, and kept at 70°C for 10 min to inactivate the enzymes. The DNA samples were extracted with phenol-chloroform and chloroform-isoamyl alcohol followed by ethanol precipitation. The DNA was then ligated and used in transformation of *E. coli* JM109 cells. A number of deletion plasmids were purified, confirmed by restriction analyses and used in sequencing.

Denaturation of Template

Approximately 1 μg of dsDNA in 20 μl solution was denatured with 0.2 N NaOH and 0.2 mM EDTA at room temperature. It was neutralized with 0.3 M Tris (pH 4.5) and 0.8 M sodium acetate on ice. The DNA was precipitated with two volumes of 100% ice-cold ethanol, washed with 70% ethanol and vacuum dried. The denatured DNA was dissolved in distilled water for immediate use or stored at -20°C until needed.

Single-stranded DNA template (about 1 μg) was denatured by heating at 95°C for 3 min and used immediately.

Annealing of Template to Primer

A number of synthetic primers obtained from Recombinant DNA/Protein Resource Facility, Department of Biochemistry and Molecular Biology, Oklahoma State University, as well as M13 reverse and forward primers (Promega) were used in DNA sequencing. A 10 μl annealing reaction containing template, primer (3 ng per assay) and 1x annealing reaction buffer [200 mM Tris-HCl (pH 7.5), 100 mM $MgCl_2$, 250 mM NaCl, and 0.1 M dithiothreitol (DTT)] was incubated at 37°C to allow annealing of primer to dsDNA template for 20 min essentially as described by the supplier (USBC). The same annealing reaction was set up for ssDNA templates except that annealing was done at room temperature for 30 min.

Extension and Labeling

To each annealed template-primer mixture, 1 μl of DTT (0.1 M), 2 μl of labeling nucleotide mix [1.5 μM dGTP, 1.5 μM dCTP, and 1.5 μM (α–^{35}S) dATP (5 μCi)] and 2 μl (3 units) of Sequenase Version 2.0 enzyme were added to attain a total volume of 15 μl. The extension reaction was performed on ice for 10 min.

Termination

Four prewarmed microfuge tubes labeled as "G", "A", "T", and "C" were filled with 2.5 μl the appropriate dideoxy (ddNTP) termination mixture

provided by the manufacturer. When the extension reaction was complete, 3.5 μl of the labeled reaction mixture was aliquoted to each of the four tubes. The contents were mixed by brief centrifugation. After 4 min of incubation at 49°C, the reaction was terminated by addition of 4 μl stop solution (95% formamide, 20 mM EDTA, 0.05% bromophenol blue and 0.05% xylene cyanol FF). After heat denaturation at 90°C, 2 to 3 μl of samples were loaded in each lan, the remaining samples were stored at -20°C.

Denaturing Gel Electrophoresis

Before loading the samples, the gel was prerun using 0.5x TBE in the top and 1x TBE in the bottom chamber for about one h or until the temperature reached 45°C. Samples were then loaded and the electrophoresis was continued at 35 watts constant power. Depending on the length of the sequence to be obtained, some samples were electrophoresed in a staggered manner one after another. To get effective resolution of the sequence 3 M sodium acetate was added to the bottom chamber buffer to a final concentration of 1 M after the bromophenol blue in the samples ran off. After electrophoresis, the gel apparatus was dismantled, and the glass plate with gel was fixed in 10% glacial acetic acid solution with occasional agitation at room temperature for one h. The gel was briefly rinsed with distilled water and dried at 65°C in a dry air oven. The gel was exposed to X-ray film (Kodak XAR-5) under the safety light at room temperature. The film was developed and fixed according to the manufacturer's recommendations.

Analyses of Nucleotide Sequences

Nucleotide sequences were compared for similarity with other DNA sequences in different data banks using the MacVector 3.5 software (IBI). The deduced amino acid sequences of the open reading frames were also compared with similar sequences in the data bases via University of Oklahoma Computer Center. The Sequence Analysis Software Package (Genetics Computer Group, Inc., Wisconsin) was also used to analyze DNA sequences for homologies, restriction analysis, alignment of open reading frames and amino acid sequences.

CHAPTER IV

CHARACTERIZATION OF A NOVEL CONJUGATIVE TRANSPOSON, Tn*5251*, FROM ΩBM6001 ELEMENT OF *STREPTOCOCCUS PNEUMONIAE.*

RESULTS AND DISCUSSION

Instability of *tet* Element in *E. coli:*

A 23.2-kb *Xba*I fragment containing the *tet* determinant from the central region of Tn*5253* (Figure 1) was cloned into *Xba*I digested *E. coli* vector plasmid pVA891 to create the recombinant plasmid pVJ403. pVJ403 was very stable in both recombination deficient and recombination proficient *E. coli* hosts if tetracycline selection was maintained (3). However, when grown in the absence of tetracycline, pVJ403 gave rise to the deletion derivative plasmid, pVJ403Δtet. Restriction endonuclease analysis of the plasmids from a number of tetracycline sensitive (Tc^S) isolates showed that in every plasmid an 18-kb fragment internal to the passenger DNA was deleted. The restriction endonuclease maps of the parental plasmid, pVJ403, and its deletion derivative, pVJ403 Δtet, are shown in Figure 2. The deletion was mapped to lie between the *Hin*dIII site 3.0 kb away from the left end of the passenger DNA and the *Bam*HI site 1.4 kb from the right end.

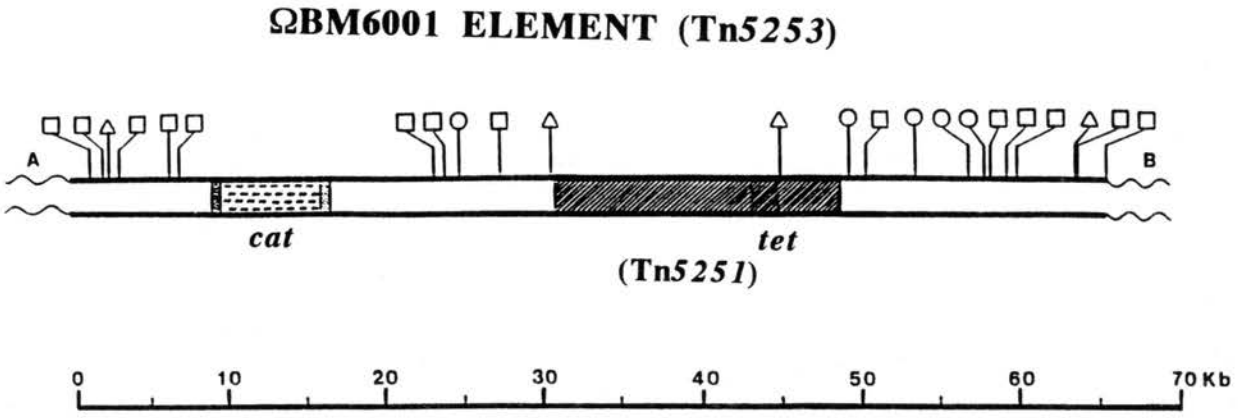


Figure 1. Physical Map of Tn5253. A and B indicates pneumococcal chromosome. ▭, *cat* determinant; ▮, direct repeats flanking the *cat* determinant; ▨, Tn*5251* DNA; ⚲, *Xba*I; ⚲, *Kpn*I; ⚲, *Bam*HI.

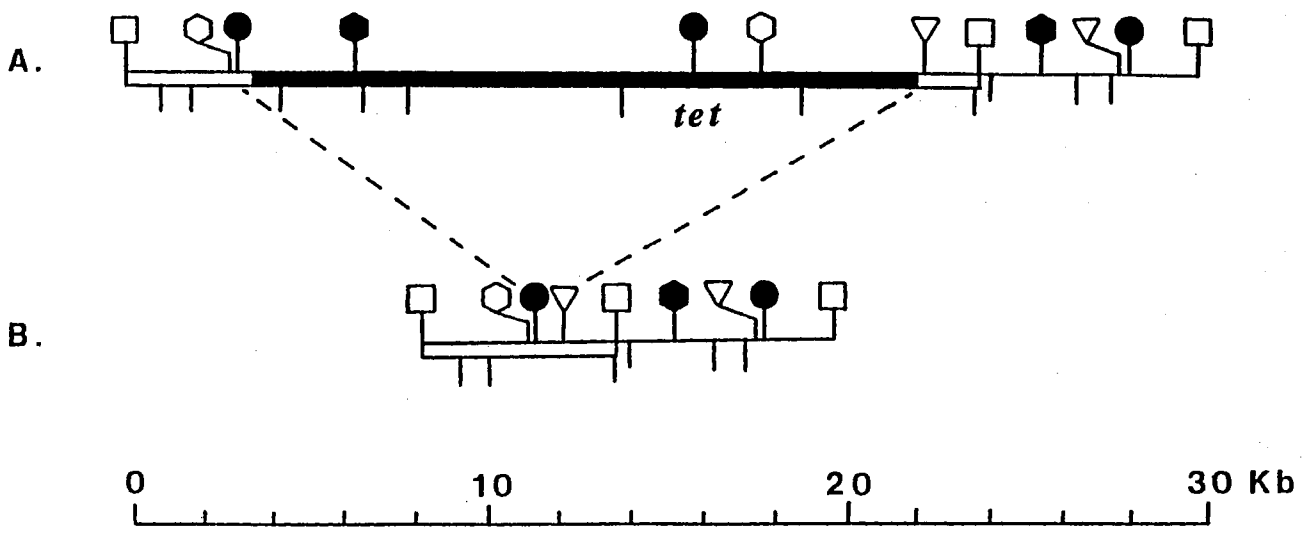


Figure 2. Restriction Endonuclease Map of pVJ403 and Deletion Derivative, pVJ403Δ*tet*. A, pVJ403; B, pVJ403Δ*tet*. ⊓, *Xba*I; ⬡, *Kpn*I; ▽, *Bam*HI, ●, *Hin*dIII; ⬢, *Eco*RI; ⊤, *Hin*cII. The shaded region indicates Tn*5251*.

The resulting *Xba*I fusion fragment in all tested deletion derivative plasmids was found to be 5.2 kb suggesting a precise excision of the *tet* determinant.

Similar observations with two other related conjugative tranposons have been previously reported (11, 27). *S. pneumoniae* BM4200 strain carries a transferable resistance to Cm, Em,Km and Tc (8, 21). When a part of it was cloned in *E. coli*, a 25-kb segment was observed to excise from the plasmid replicon and to insert into *E. coli* chromosome in the absence of selection. The transposing part of the BM4200 element, designated Tn*1545*, exhibited properties of intercellular and intracellular transposition when it was introduced in *E. faecalis* cells by transformation (22). Likewise, a conjugative *tet* element, Tn*916*, first discovered in the chromosome of *E. faecalis* DS16 (26), was able to transpose intracellularly from the chromosome to the hemolysin plasmid, pAD1, and to a number of species intercellularly via conjugation. When an *Eco*RI fragment carrying the Tc^r from the hemolysin plasmid pAD1 was cloned in *E. coli*, a 16-kb DNA segment excised from the chimeric plasmid and resulted in regeneration of an insertionally activated streptococcal DNA fragment (27).

Transposition of the *tet* Element from pVJ403 in Pneumococci

Excision of the *tet* element from pVJ403 in *E.coli* suggested that the element may undergo transposition when it is physically separated from the parental conjugative transposon Tn*5253*. However, independent movement of an 18-kb *tet* element, when present in pneumococcal chromosome as a part of the larger transposon Tn*5253* (Figure 1), has not been reported (66).

To determine whether the excision of the *tet* element from pVJ403 was due to a transposition event, this plasmid was used as donor DNA in transformation of pneumococcal Rx1 cells. As donor DNA enters pneumococci during transformation in a single-stranded form (48, 49), plasmid establishment would require entry of two overlapping complementary molecules and subsequent generation of an intact circle (52, 60). Since the vector portion of pVJ403 was incapable of autonomous replication in streptococci (45) and the recipient cells lacked homology to the donor DNA, no Em^r or Tc^r transformants were expected by normal genetic recombination. As expected no Em^r transformants were found. However, more than 40 Tc^r transformants per $2x10^7$ CFU per 10 μg of plasmid DNA were obtained (Table IV). All Tc^r transformants were eryhtromycin sensitive (Em^s), indicating that the vector was lost, and that the insertion of the heterologous *tet* element did not involve the homology dependent insertion-duplication pathway (76).

To confirm the insertion of the *tet* element into the Rx1 chromosome, chromosomal DNAs from 10 Tc^r transformants were used as donor DNA in a second-round of transformation of Rx1 cells. If the *tet* element had inserted in the chromosome, transformation efficiency of the recipient cells with tetracycline should have been much greater due to the homologous sequences flanking the *tet* element. The Tc^r transformants were observed at least four orders of magnitude higher (Table V) indicating that the *tet* insertions were chromosomal.

For further confirmation of chromosomal *tet* insertions, chromosomal DNAs from the same Tc^r strains were used in complementation experiments with DP1333 strain, carrying a *tet-3* point mutation and sensitivity to chloramphenicol (Cm^s) due to spontaneous loss of the *cat* gene (77).

TABLE IV

TRANSFORMATION OF Rx1 WITH pVJ403
CARRYING THE *tet* ELEMENT

	Transformants/ml		
Donor DNA	Tc^r	Em^r	Str^r
pVJ403	43	<10	
pAM118 [a]	110	100	
DP1617 [b]			$1.1x10^5$

[a] pAM118 (pVA838::Tn*916*, Em^r, Tc^r, ref. 27) was used as a control.

[b] DP1617 DNA (see Table I) was used as a control for determining the competence level of Rx1 recipient cells.

TABLE V

CONFORMATION OF Tn*5251* INSERTION IN PNEUMOCOCCAL CHROMOSOME BY TRANSFORMATION

	Tc^r transformants/ml	
Donor DNA	Rx1	DP1333
OK 1	$4.7x10^4$	$2.5x10^5$
OK 2	$3.6x10^4$	$4.5x10^5$
OK 3	$1.2x10^5$	$4.7x10^5$
OK 4	$8.7x10^4$	$4.8x10^5$
OK 5	$6.3x10^4$	$5.0x10^5$
OK 6	$2.1x10^5$	$6.0x10^5$
OK 7	$9.2x10^4$	$5.2x10^5$
OK 8	$5.1x10^4$	$6.3x10^5$
OK 9	$4.7x10^4$	$4.7x10^5$
OK 10	$2.9x10^4$	$3.6x10^5$

Transformation efficiency of Rx1 and DP1333 with *str-1* chromosomal marker from DP1617 was 3.2 $x10^6$ and $1.2x10^6$ transformants per ml respectively.
Spontaneous Tc^r and Em^r mutants were less than ten per ml for both recipient strains.

The efficiency of Tc^r transformants was found to be five orders of magnitude greater with reference to spontaneous Tc^r revertants, no Em^r transformants of DP1333 were obtained (Table V).

To determine whether the transposition of the *tet* element during transformation involved unique or multiple target sites, eight of Tc^r transformants were analyzed in blot hybridization experiments. *Eco*RI digests of chromosomal DNA were probed with pVJ403 and pVJ403Δtet. In each case, two fragments of varying sizes strongly hybridized to the probe, pVJ403, representing the chromosome-*tet* element junction regions indicating the random insertion of the *tet* element (Figure 3). Furthermore, pVJ403Δtet, which contained the same segment of DNA as pVJ403 with the exception of the *tet* element, did not react with the clones. This implies that the transposing part did not carry any of the flanking DNA detectable at this level of resolution. In addition, blot hybridization indicated that in each clone a single copy of the *tet* element had inserted into the chromosome of the Rx1 strain.

Intraspecific Conjugative Transfer of the *tet* Element

To test whether the *tet* element was capable of mobilizing the tetracycline resistance to pneumococcal recipients by conjugation, two Tc^r clones SP1704 (Figure 3, lane a) and SP1705 (Figure 3, lane b) were used as donor in filter-mating experiments with DP1004 (Rx1/*str-1*) recipients. Before the filter mating, a chromosomal novobiocin resistance gene (*nov-1*) was introduced into these two Tc^r clones in order to facilitate the selection of transconjugants. Transconjugants were selected on CAT agar containing

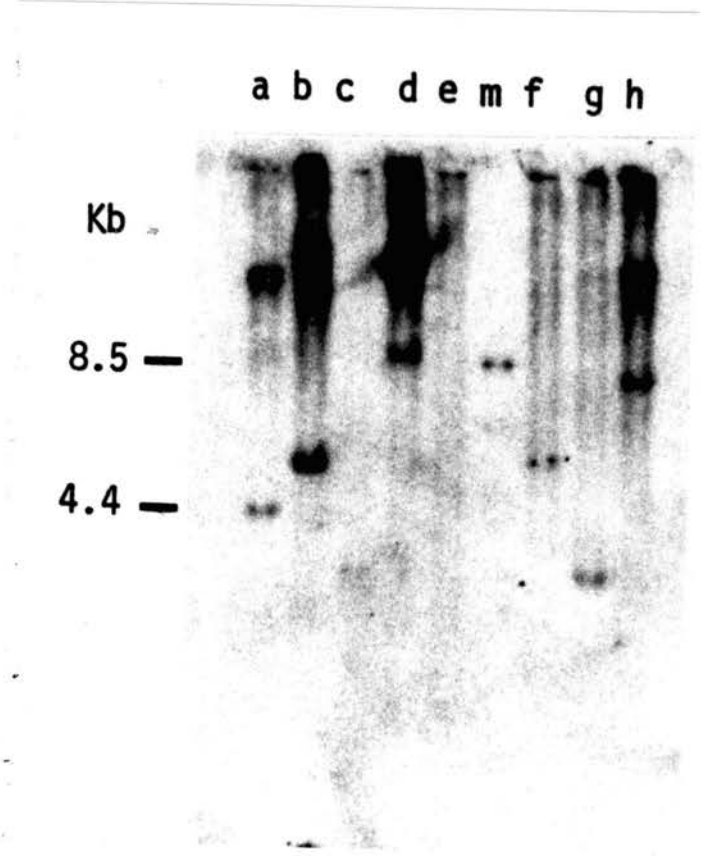


Figure 3. Autoradiogram Showing the Transposition of the *tet* Element into Pneumococcal Chromosome. *Eco*RI-digested chromosomal DNAs from eight Tc^r transformants (lane a through h) probed with radioactively-labeled pVJ403, lane m indicates molecular weight standard.

streptomycin and tetracycline. Both SP1704 and SP1705 were able to transfer the Tc^r at a frequency of 10^{-5} per donor, a frequency comparable to that of the control strain DP1355 (*nov-1*, Tn*916*), under conditions where transfer of the chromosomal marker, *nov*, could not be detected (TableVI). These results suggest that an18-kb segment of DNA carrying the *tet* determinant (designated Tn*5251*) had features of a conjugative transposon: an ability to excise independently and insert into the chromosome of pneumococci in a Rec^- environment in the absence of any mobilizing plasmid or transducing bacteriophages.

Physical Analysis of Tc^r clones of Pneumococci by Southern Hybridization

When introduced into Rx1 cells by transformation, the *tet* element excises from the vector plasmid, pVJ403, and inserts into multiple sites on the pneumococcal chromosome as indicated previously (Figure 3). In addition, the *tet* element has been shown to conjugally transfer among pneumococci (Table VI). The strain SP1704 was further in filter-mating experiments with pneumococcal recipients and transconjugants were analyzed in blot hybridization experiments. The strain SP1704 was mated with both DP1004 and SP1000 recipients carrying the *cat* element, Tn*5252* (3). The latter mating was performed to determine whether Tn*5251* inserts in the same place within Tn*5252* from which it had been deleted. Tn*5252* was constructed by *in vivo* deletion of the *tet* element within the parental element.

TABLE VI

INTRA- AND INTERSPECIFIC TRANSFER OF Tn*5251* BY FILTER-MATING

Donor	Recipient	Str^r Tc^r transconjugants per donor
SP1704	**DP1004**	3.0×10^{-5}
SP1705	**DP1004**	1.3×10^{-5}
DP1355 [a]	**DP1004**	1.6×10^{-5}
SP1704	***S. pyogenes***	1.0×10^{-6}
GP 42 [b]	***S. pyogenes***	1.1×10^{-6}

a,b: used as controls. SeeTable I.

A total of eight Tn*5251* transconjugants of SP1000 recipients obtained from three different mating experiments were probed with pVJ403 in a blot-hybridization experiment (Figure 4). In addition, five Tn*5251* transconjugants of DP1004 obtained from two separate matings were digested with *Eco*RI and probed with pVJ403. As evident from the autoradiogram, Rx1 did not react to the probe as expected (Figure 4, lanes a). DP1324 carrying Tn*5253* showed two radioactive bands of about 22 kb and 12 kb in size (lane b). A single fragment of 16.6 kb from SP1000 (lane c), and two fragments from SP1704 (lane d) reacted with the probe as expected. It is worth noting that, both SP1000 and DP1004 transconjugants carrying Tn*5251* showed the same hybridization pattern with the probe suggesting that Tn*5251* had inserted in the same site in the chromosome of SP1000 and DP1004. Moreover, it seemed that instead of inserting at the original site within Tn*5252* from which it was physically removed, Tn*5251* inserted at an alternate single site in the chromosome of SP1000 transconjugants (lanes e through n). The pattern was identical to that of the donor SP1704 strain (lane d). If Tn*5251* had inserted within Tn*5252*, the hybridization pattern of such a clone would be expected to be the same as the parental element, Tn*5253*, in DP1324 (lane b). Likewise, all in the five DP1004 transconjugants (lane o through s), contained two *Eco*RI fragments that hybridized to the probe in the same fashion as in the donor strain SP1704.

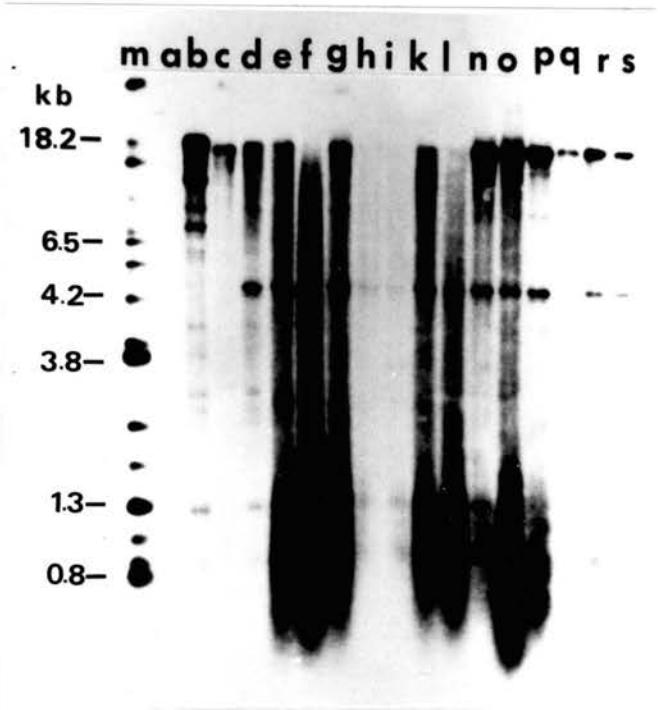


Figure 4. Physical Analyses of Tc^r Clones of SP1000 and DP1004 Recipients by Southern Hybridization. *Eco*RI cleaved chromosomal DNA from Rx1 (lane a), DP1324 (lane b), SP1000 (lane c), SP1704 (lane d), Tc^r clones of SP1000 recipients (lanes e through n), and Tc^r clones of DP10004 recipients (lanes o through s) were probed with pVJ403. Molecular weight standard (lane m).

Interspecific Conjugative Transfer of the *tet* Element

To further analyze the conjugal-transfer ability of the *tet* element, SP1704 was used as a donor in filter mating experiments with *S. pyogenes* recipients. The transconjugants were spread on CAT agar containing optochin and tetracycline (unlike pneumococci, *S. pyogenes* can grow under aerobic conditions). *S. pyogenes* transconjugants were screened for the unselected donor marker (*nov-1*) and confirmed by their cultural and phenotypic characteristics on blood agar plates. As shown in Table VI, the *tet* element was able to conjugally transfer from pneumococci to *S. pyogenes* at a frequency of 10^{-6} per donor cell. The donor ability of SP1704 was comparable to that of the control strain, GP42, under the same conditions. Transfer of the chromosomal markers, *nov-1* and *str-1*, from SP1704 and GP42, respectively, were not detected. Since natural transformation was not evident for this *S. pyogenes* strain, Tc^r clones obtained from this mating were expected to be the result of conjugation. No spontaneous Tc^r revertants of *S. pyogenes* were detected.

Physical Analysis of Tc^r clones of *S. pyogenes* by Southern Hybridization.

Chromosomal DNAs from six of the *S. pyogenes* transconjugants obtained from three different matings were digested with *Eco*RI and probed with pVJ403 in blot hybridization experiments (Figure 5). Chromosomal DNAs from Rx1 (Figure 5, lane a) and *S. pyogenes* (lane e) did not react with the probe. DP1324 carrying the parental transposon, Tn*5253*, (lane b)

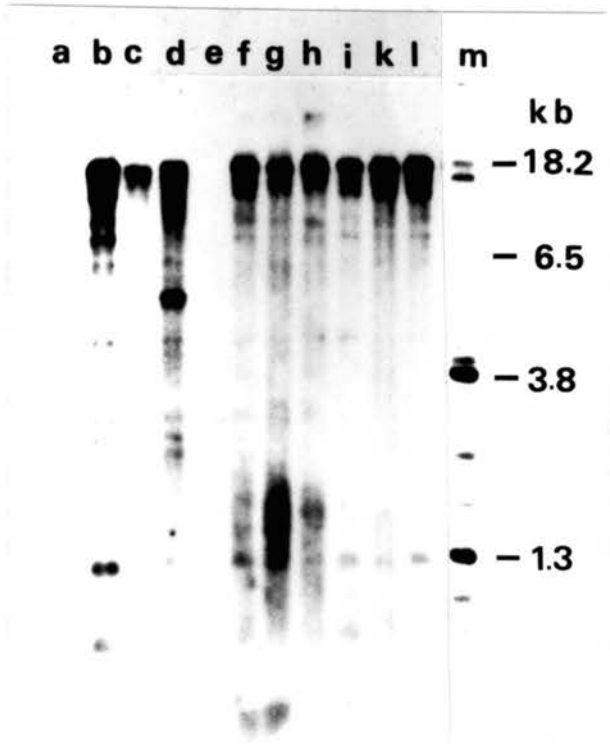


Figure 5. Physical Analyses of Tc^r Clones of *S. pyogenes* Recipients by Southern Hybridization. *Eco*RI-cleaved chromosomal DNAs from Rx1 (lane a), DP1324 (lane b), SP1000 (lane c), SP1704 (lane d), *S. pyogenes* (lane e), Tc^r clones of *S. pyogenes* recipients (lanes f through l) were probed with pVJ403. Molecular weight standard (lane m).

reacted with two *Eco*RI fragments, 22.7 kb and 12.2 kb. A single 16.6-kb *Eco*RI fragment in SP1000 (lane c) reacted to the probe as expected. SP1704 carrying the *tet* element (lane d) reacted with two *Eco*RI fragments, about 20 kb and 5 kb in size. The six Tc^r clones of *S. pyogenes* (lanes f through l) reacted to the probe confirming the conjugal transposition of the *tet* element to this species. Differences in the reaction conditions applied in this experiment seemed to result in the appearance of some background fragments in both DP1324 and SP1704. As evident from the previous autoradiogram (Figure 3, lane a), SP1704 DNA showed only two *Eco*RI fragments (20 kb and 5 kb) reacted with the radioactively labeled pVJ403. Presence of at least four extra fragments (less than 3 kb) of SP1704 (Figure 4, lane d) might have been due to the result of star activity of *Eco*RI enzyme. The poor quality of the restriction enzyme digest complicated the analyses of these Tc^r transconjugants whether a single or multiple copies of the *tet* element were present on the chromosome of *S. pyogenes*. However, this experiment indicated that the *tet* element had transferred to *S. pyogenes* recipients via conjugation. All six samples hybridized with very similar, if not identical, *Eco*RI fragments indicating that insertion of the *tet* element into the chromosome of *S. pyogenes* was not random.

As mentioned previously, Tn*5251* shows extensive structural (43) and functional similarities to those of related *tet* elements and inserts randomly in the chromosome of pneumococci. However, the larger element, Tn*5252*, differs from Tn*5251*- like transposons and behave more like the parental element, Tn*5253*, with respect to its target specificity (3). These results, however, were insufficient and it remained unclear whether Tn*5251* has a preferred target site in both pneumococci and *S. pyogenes* (Figures 4 and 5).

The possibility of a transformation event was unlikely because of the presence of DNase during the mating. Furthermore, the absence of the donor marker (*nov-1*) in the transconjugants indicated that transformation did not take place. The results obtained from the blot hybridization experiments (Figures 4 and 5) could also rule out the possibility of the presence of a cryptic plasmid in the donor strain on which Tn*5251* had inserted. If this was the case, both *S. pyogenes* and pneumococcal recipients would have had identical hybridization pattern. However, this seemed not to be the case because *S. pyogenes* transconjugants reacted with at least two fragments (Figure 5) with different sizes than those of the donor and pneumococcal transconjugants (Figure 4).

The transfer of Tn*5251* to a single site on the chromosome of both pneumococci and *S. pyogenes*, could be possible if it resides on a cryptic chromosomal fertility factor which has a target preference for insertion in these two species. Since only one pneumococcal strain carrying Tn*5251* was used as a donor in the filter-mating experiments, it is not possible to conclude whether the transfer of Tc^r was due to transposon-encoded conjugative functions or to a fertility factor into which Tn*5251* had been inserted. To test this, other donor strains carrying Tn*5251* insertions at different locations could be used in filter-mating experiments with the recipients of the same species. In addition, a transfer-deficient derivative of Tn*5251* with a selectable marker would be used to replace the wild type transposon in strain SP1704 by transformation. The subsequent strain could then be tested for its ability to donate transfer deficient Tn*5251* derivative via conjugation. The conjugal transfer of the mutant transposon would be expected only if there is a fertility function in the donor strain. Bringel *et al.* (5) reported the existence of a chromosomal fertility factor in *Lactococcus*

lactis subsp. *lactis* which was able to act as a donor of both chromosomal markers and Tn*916* to the derivatives of *L. lactis* MG1363 strain.

Co-existance of Tn*5251* with a Conjugative Plasmid

Most streptococci, except for pneumococci, carry drug resistance plasmids. However pneumococci have the ability to acquire and maintain these plasmids from other streptococci and related bacteria. Moreover, they are able to donate these plasmids to other streptococcal species at a frequency of 10^{-6} to 10^{-3} per donor cell via conjugation. Conjugative plasmids of streptococcal origin (such as pAMβ1 and pIP501) have been shown to be capable of mobilizing a nonconjugative plasmid, pMV158, conferring tetracycline resistance (56). The broad host range conjugative plasmid pIP501, 30.2 kb in size, originally isolated from *S. agalactiae* confers resistance to erythromycin and chloramphenicol (36).

To understand the interaction between conjugative transposon and conjugative plasmid, three strains (SP1704, SP1705 and SP1706) carrying Tn*5251* were mated with DP3220 (carrying pIP501). DP1002 (Rx1/*nov-1*) strain was also used as a control in the conjugation experiment with DP3220 strain (Table VII). Transconjugants were selected on CAT agar plates containing combination of two antibiotics to score the exchange of the drug markers among two strains. For example, Tn*5251* transconjugants of DP3220 recipients were selected in the presence of streptomycin and tetracycline, and pIP501 transconjugants in the presence of novobiocin and erythromycin. In addition, all transconjugants were screened for unselected markers by replica plating. Transfer frequency of pIP501 and Tn*5251* was

TABLE VII

CONJUGAL TRANSFER FREQUENCY OF Tn*5251* TO A PNEUMOCOCCAL RECIPIENT HARBORING A CONJUGATIVE PLASMID

Parental Strains	Transconjugants/ml	
	Str^r/Tc^r	Nov^r/Cm^r
DP3220 X SP1704	2.0×10^{-5}	$< 3.1 \times 10^{-7}$
DP3220 X SP1705	1.0×10^{-5}	3.0×10^{-5}
DP3220 X SP1706	4.6×10^{-5}	7.1×10^{-5}
DP3220 X DP1002	$< 3.6 \times 10^{-7}$	1.8×10^{-5}

found to be 10^{-5} per donor cell. The transfer of pIP501 from DP3220 to SP1704 was not detected ($< 3.1 \times 10^{-7}$ transconjugants per ml). This was found to be as a result of spontaneous loss of novobiocin resistance in strain SP1704. These results suggested that Tn*5251* is capable of co-existing with the conjugative plasmid pIP501. In other words, neither Tn*5251* nor pIP501 had any type of entry-exclusion function or immunity that inhibits the mobilization of the other element.

Physical Map of Tn*5251*

The recombinant *E. coli* plasmid, pVJ403 (Figure 2), carrying Tn*5251*, was digested with a number of restriction endonucleases and the resulting restriction fragments were analyzed following electrophoresis on 0.8% agarose gel. The *tet* gene of Tn*5251* was mapped to lie within a 5-kb *Hin*cII fragment. Of the sixteen restriction enzymes *Ava*I, *Bam*HI, *Bgl*II, *Hae*III, *Sal*I, *Sph*I, *Pst*I and *Xba*I did not have recognition sites within the *tet* element. Using sixteen different restriction enzymes, the physical map of Tn*5251* was constructed (Figure 6).

The restriction map of Tn*5251* showed significant similarities to the *tet* transposons Tn*916* and Tn*1545* (43). In both Tn*916* and Tn*5251* a single *Hin*dIII restriction site lies within the *tetM* gene, and both Tn*916* and Tn*5251* lack *Hae*III restriction site.

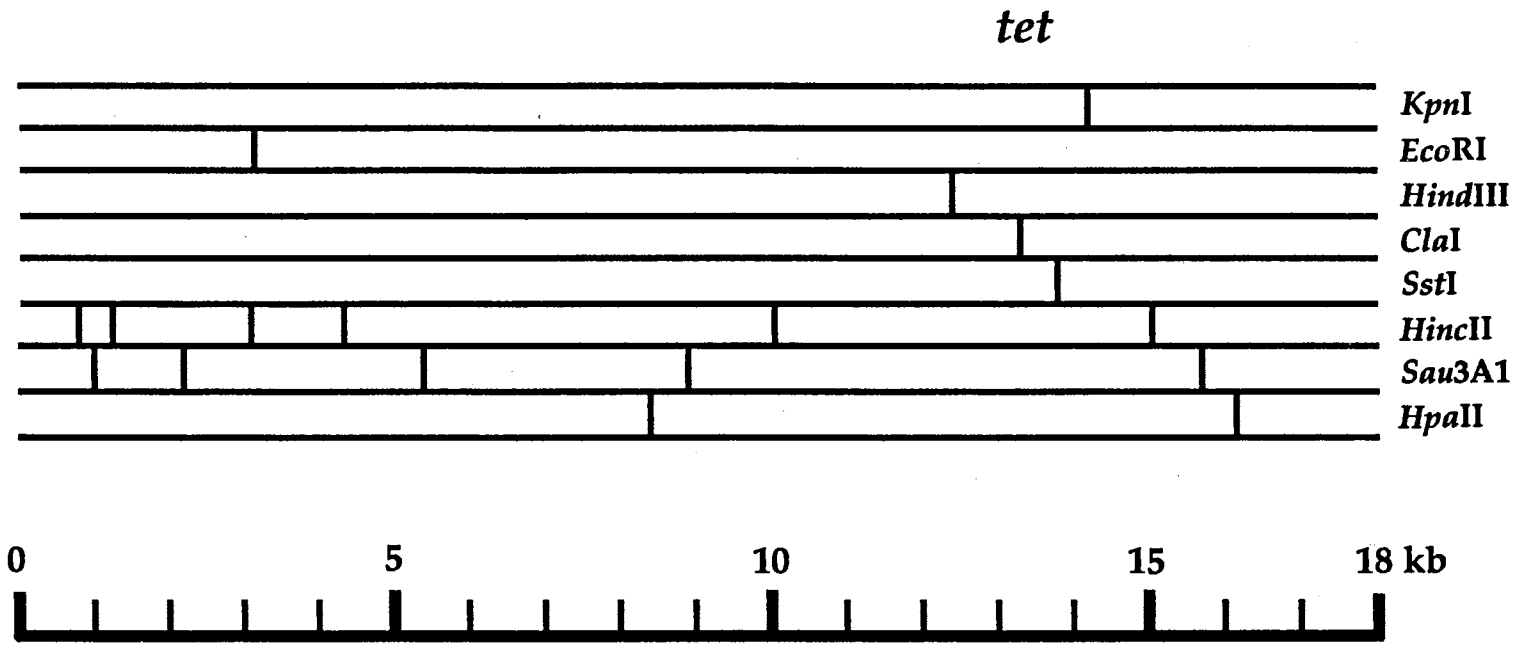


Figure 6. Physical Structure of Tn*5251*

The aim of this study was to investigate the origin, evolution, and genetics of the streptococcal conjugative ΩBM6001 element.
The ΩBM6001 element originally found in *S. pneumoniae* is a 65.5-kb conjugative element that carries resistance to chloramphenicol and tetracycline. By specific insertion of a heterologous *E. coli* vector plasmid, pVA891, within the element, a number of recombinant plasmids carrying the entire transposon have been generated (77). The recombinant plasmid, pVJ403, carrying the *tet* region from the transposon, was constructed in *E. coli*. In the absence of tetracycline selection the apparent loss of an 18-kb DNA fragment containing the *tet* determinant from the recombinant plasmid, pVJ403, suggested a possible transposition event. To determine whether the excision of the *tet* determinant in *E. coli* was related to transposition, this recombinant plasmid was used as donor DNA to transform wild-type pneumococcus strain, Rx1.

Based on several lines of evidence, it has been shown that the *tet* determinant (now termed Tn*5251*) of the ΩBM6001 element is an independent conjugative transposon within the larger conjugative transposon (termed Tn*5253*). This novel transposon is capable of conjugation and transposition when removed from the context of the larger parental transposon.

Tn*5251* can excise and transpose in *E. coli*, and is able to integrate into the chromosome of pneumococci independently from the host recombination system. Therefore, Tn*5251* has properties of a conjugative transposon by virtue of its ability to transfer among streptococcal strains in the absence of any mobilizing plasmid or bacteriophages.

In this study, it has been shown that Tn*5251* has extensive functional and structural similarities to Tn*916* and related conjugative transposons

which have been reported elsewhere (43). Tn*5251* was found to be similar to other transposons of the Tn*916*-class including Tn*920*, Tn*925*, Tn*3702* from *E. faecalis*, Tn*919* from *S. gordonii*, Tn*3703* from *S. pyogenes*, Tn*5031* from *Enterococcus faecium*, and Tn*1545* from *S. pneumoniae* (20). Similarities between Tn*5251* and these other *tet* elements suggest a common ancestry.

The presence of *tet* determinants among a variety of streptococci raises questions concerning the evolution of these transposons. The hypothesis has been raised that a *tet*-containing transposon similar to Tn*916* was the prototype element for all other conjugative transposons. They, in turn, have served as a receptacle for the accumulation of other heterologous drug-resistance determinants resulting in increases in size (18, 31). However, when Tn*916* was used as a probe with the recombinant plasmids derived from the various regions of the Tn*5253* element, the probe reacted to only those fragments from the *tet* region but it failed to hybridize with the junction fragments of Tn*5253*.

A second study reported by Le Bouguenec and coworkers (43) compared three composite conjugative transposons, Tn*3701* of *S. pyogenes* A454, Tn*3951* of *S. agalactiae* B109, and Tn*5253* of *S. pneumoniae* BM6001 by DNA-DNA hybridization. In each case, the Tn*916*-like region carrying *erm*, as well as *tet*, and shared significant homology to the central regions of these composite elements. These findings suggested that, rather than being a prototype, Tn*916*-like sequences were added later to a preexisting element giving rise to a composite structure.

S. pneumoniae BM4200, carrying transferable resistance to chloramphenicol, erythromycin, kanamycin, and tetracycline is more than 60 kb (71). However, when a part of this larger element was cloned in *E. coli*,

a 25-kb segment carrying *erm, aphA* and *tet* resistance determinants had excised as an independent unit (21, 22). When introduced into *E. faecalis*, this segment, Tn*1545*, like Tn*916*, exhibited properties of intracellular transposition and subsequent interspecific conjugation to a number of species.

In this study, I was able to show that the *tet* element designated Tn*5251*, from the central region of Tn*5253*, was capable of transposition from the recombinant *E. coli* plasmid into the pneumococcal chromosome randomly. In addition, this transposon transferred to *S. pyogenes* as well as to pneumococcal recipients at a frequency of about 10^{-6}-10^{-5} per donor cell in filter mating.

These results suggested that, Tn*5251* belong to the Tn*916*-class transposons and rather than being progenitors of the composite conjugative transposons such as Tn*3701*, Tn*3951* and Tn*5253*, Tn*916*-like elements were probably added to the larger elements at a later time in the evolutionary process.

It was evident from the hybridization studies (43) that, Tn*5251* and Tn*916* showed DNA homology in the sizes of four internal *Hin*cII fragments, and the location of *tetM* gene resides on a 4.8-kb *Hin*cII fragment of Tn*916*. As in Tn*916*, the *tetM* gene of Tn*5251* was mapped within a 5-kb *Hin*cII fragment and a single *Hin*dIII site falls within the *tetM* gene (Figure 6). Similarities between Tn*5251* and Tn*916* suggested that they probably share a similar mechanism of excision-integration and transfer.

Among these related transposons, only Tn*916* and Tn*1545* have been characterized in detail. Genetic and sequence analyses revealed that the Tn*916*-like transposons appear to be related to the lambdoid bacteriophages in that they excise and integrate by a Campbell-like reciprocal-

recombination mechanism (20). The integrase (*int*-Tn) and excisinase (*xis*-Tn) proteins encoded by these transposons exhibit significant homology with those of lambdoid bacteriophages such as P22, lambda, and Φ80 (20). The ends of Tn*916* and Tn*1545* share at least 250 identical bases with short imperfect and direct repeats (19). Unlike the nonconjugative transposons, integration of Tn*916*-like conjugative transposons does not give rise to a duplication of the target sequence (18). On the other hand, upon excision of the transposon, the original target sequence is altered. The transposition event involves a covalently-closed-circular intermediate produced by excision of the transposon from the donor DNA (10, 61). Following staggered nicks on each strand, about five-to-six bases, at the end of the transposon, the ends are ligated together to produce a heteroduplex-circular transposon and heteroduplex target molecule. The mismatched bases "coupling sequences" at the target are resolved following replication. The insertion of the excised molecule introduces a coupling sequence into its new target derived from its previous target. Unlike lambda, integration of Tn*916*-like transposon is not completely random and the event is not site specific. The conjugative transposons lack immunity or entry exclusion in contrast to phage lambda. However, it has been shown that Tn*916* inserts at a preferred site in the chromosome of some recipient strains. When transferred from *E. faecalis* to *S. mutans* 6715 strain, Tn*916* inserted reproducibly at the same spot (18). Preference of a "hot spot" for Tn*916* into *Bacillus stearothermophilus* BR219 following its conjugal transfer from an *E. coli* donor strain has been reported (50). The Tn*916*-related conjugative transposon Tn*919* has been shown to insert at a single site on the chromosome of *L. lactis* MG13416 strain following its conjugal transfer (33).

The data presented in this study indicated that the insertion sites of Tn*5251*-type transposons may be influenced by some host factors which are yet to be determined. In addition, these data suggest that the insertion of Tn*5251* in pneumococci and *S. pyogenes* following conjugal transfer may involve a preferred target site. To examine the insertional behaviors of Tn*5251*, several filter matings with different recipients should be performed.

Several attempts to transfer Tn*5251* to *E. faecalis*, *B. subtilis* and *E. coli* via conjugation were unsuccessful. On the other hand, when *E. faecalis* UV202 strain carrying the parental transposon Tn*5253* was used as a donor in filter matings with pneumococcal recipients, only Tc^r Cm^s transconjugants were obtained. Eight of these transconjugants were analyzed in blot hybridization and probed with pVJ403. The data indicated that only the Tn*5251* part of Tn*5253* transferred back to pneumococci (Ayoubi, personal communication). No detectable reaction was observed with the two probes derived from the termini of Tn*5253* suggesting that Tn*5251* separated from Tn*5253* and transferred independently. Variations in the sizes of *Eco*RI fragments reacting to the probe indicated a random insertion of Tn*5251* in each of eight Tc^r Cm^s transconjugants. At least four of the transconjugants seemed to have acquired two copies of Tn*5251* .

These results indicate that some sequences flanking Tn*5251* in Tn*5253* may dictate the excision and insertion properties of the *tet* element. Also, some host factors may directly interfere with the transposition and conjugal transfer of Tn*5251*. This may be the reason that the *tet* element did not transpose or conjugally transfer independently when it was part of the parental Tn*5253* element. To understand the interactions between Tn*5251* and Tn*5252*, sequence analysis of the termini and junction fragments of Tn*5251* should be undertaken in a number of transconjugants.

Although Tn*916* and related transposons have been observed primarily in streptococci, they have an extremely broad host range. They have been mobilized into a number of species including *E. coli* and an anaerobic gram-negative bacterium, *Fusobacterium nucleatum*, as well as many other gram-positive bacteria by conjugation or transformation. Tn *916* and related transposons are able to mobilize nonconjugative plasmids at a detectable frequency (20).

Conjugative transposons, originally detected in clinical isolates, clearly play an important role, not only in the transmission of multidrug resistance among human pathogens, but also in bacterial evolution. Their ability to transfer is not limited to the clinically important strains of gram-positive species or to a single genus, they are also able to transfer horizontally from gram-positive species to gram-negative bacteria (20).

CHAPTER V

IDENTIFICATION AND LOCALIZATION OF TRANSFER-RELATED REGIONS IN THE CONJUGATIVE TRANSPOSON, Tn*5252*.

RESULTS AND DISCUSSION

Strategy for Creating Insertion/Deletion Mutations within Tn*5252*.

Construction of a set of derivatives of pVA891 carrying various segments spanning the entire Tn*5252* DNA (76, 77) facilitated introduction of deletions and insertions within the passenger fragments. The restriction map of Tn*5252* and coordinates of eleven restriction sites in Tn*5252* are given in Figure 7 and Table VIII respectively. DNA restriction fragments ranging from 1.5-to-10 kb from the passenger segments of these plasmids were isolated and subcloned into pUC-based *E. coli* plasmids. A unique site for another restriction enzyme present in these fragments was used to insert a drug-resistance gene. Also, specific DNA fragments with more than one restriction site internal to the passenger DNA fragment were subcloned into pVA891 (Figure 8) for deletion mutagenesis. The recombinant plasmids used in insertion/deletion mutagenesis are given in Figure 9 and Table IX.

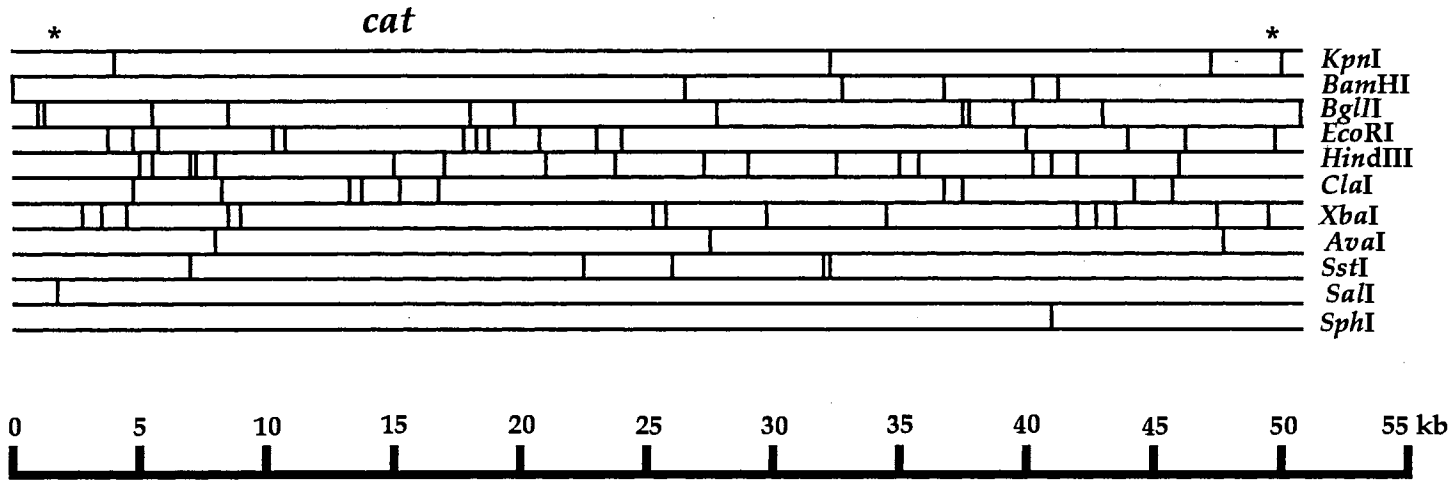


Figure 7. Physical Map of Tn*5252*, reconstructed from the phsical map of Tn*5253* Vijayakumar *et al.* (77). The asterisks indicate end of the transposon.

TABLE VIII

RESTRICTION ENDONUCLEASE SITES IN Tn*5252* [a]

*Bam*HI	*Kpn*I	*Bgl*II	*Eco*RI	*Hin*dIII	*Cla*I	*Xba*I	*Ava*I	*Sst*I	*Sph*I	*Sal*I
0.0	4.1	0.8	0.1	5.2	4.8	2.8	7.9	7.1	41.4	1.7
26.3	32.1	1.3	3.6	5.5	8.0	3.5	27.4	22.3		
33.0	47.3	5.5	4.7	6.9	13.0	4.6	47.6	25.7		
37.2	50.2	8.3	5.8	7.0	- b	8.0		31.7		
40.5		18.0	10.4	8.0	13.7	8.5		31.9		
41.6		19.5	11.0	14.8	15.8	24.6				
		27.4	17.7	17.1	16.3	25.1				
		37.9	18.1	20.9	37.0	29.2				
		38.1	18.7	23.6	37.7	34.4				
		39.8	22.7	28.6	44.4	40.6				
		42.7	23.6	32.2	- c	43.5				
		50.6	40.2	35.2	46.0	47.5				
			44.0	36.1		49.2				
			46.4	40.6						
			49.7	41.4						
				42.3						
				46.2						

[a] Distances in kb from a *Bam*HI site designated 0.0. Four pairs of close sites have been ordered as follows: *Hin*dIII sites are to the left of a *Bgl*II site near 5.5, a *Cla*I site near 8.0, and a *Sph*I site near 41.4; a *Bgl*II site is to the left of the *Ava*I site near 27.4.

[b] Another *Cla*I site in this interval is 0.24 kb from the nearest site.

[c] Another *Cla*I site in this interval is 0.14 kb from the nearest site.

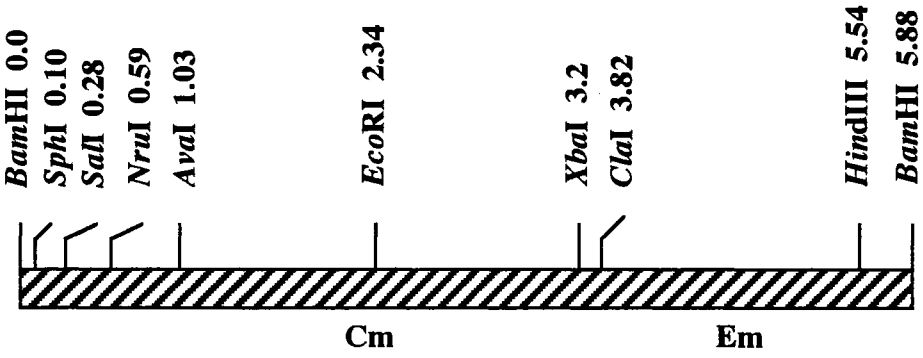


Figure 8. Restriction Map of Plasmid pVA891 (45).

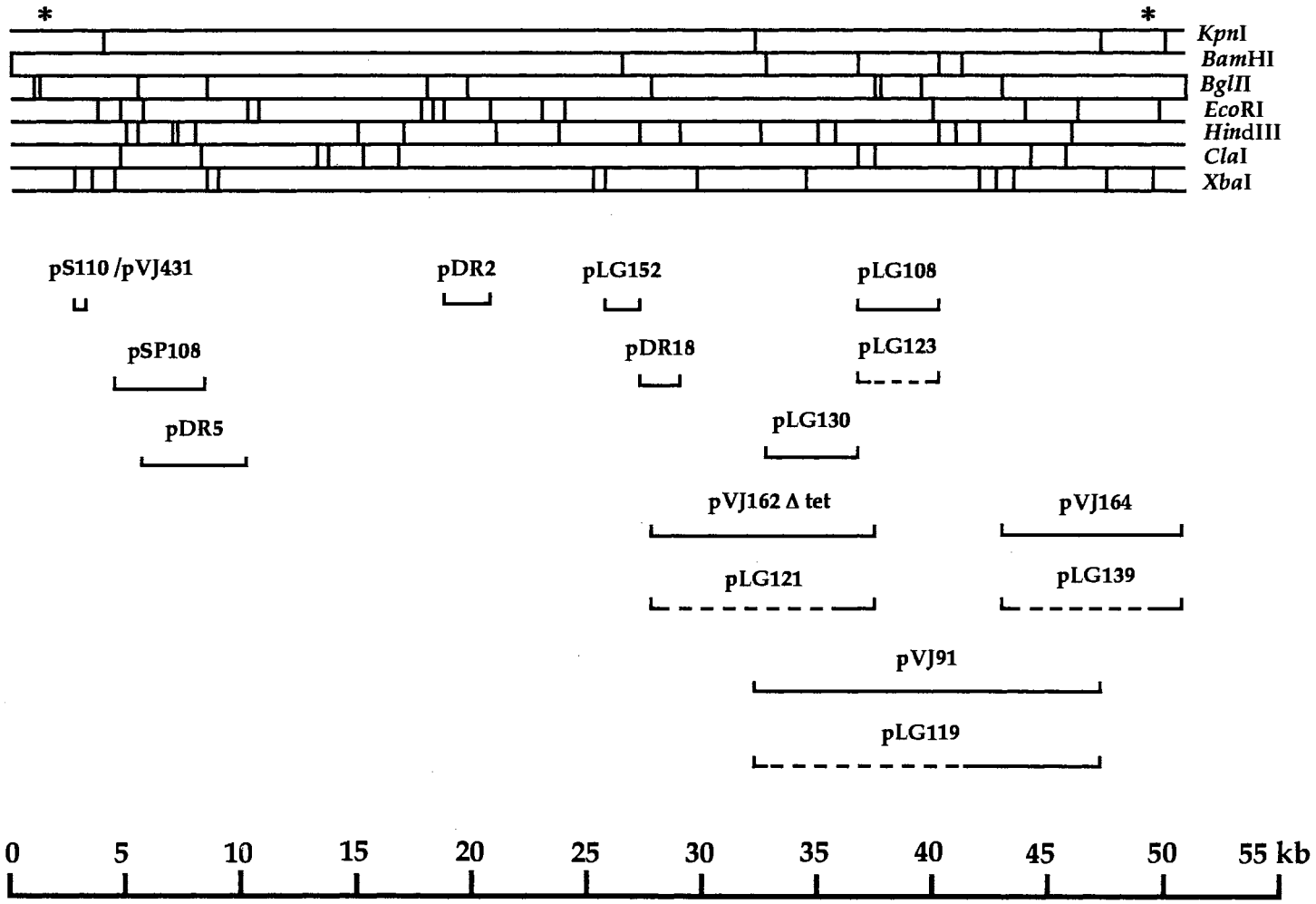


Figure 9. Recombinant Plasmids Derived from Tn*5252* Used in Deletion/Insertion Mutagenesis.

TABLE IX

RECOMBINANT PLASMIDS USED TO CREATE DELETION/INSERTION MUTANTS OF Tn*5252*

Plasmid	Transposon DNA coordinates	Restriction enzymes within the transposon used to create the plasmids	Deletion /Insertion [a] coordinates		Mutant strain created
pSS141 [b]	4.1-32.1	*Kpn*I	5.2-28.6	(*Hin*dIII)	SP1200
pSS141 [b]	4.1-32.1	*Kpn*I	4.8-8.0	(*Cla*I)	SP1201
pVJ162Δ*tet*	27.4-37.9	*Bgl*II	28.6-36.1	(*Hin*dIII)	SP1202
pVJ91	32.1-47.3	*Kpn*I	33.0-41.6	(*Bam*HI)	SP1203
pLG108	37.2-40.5	*Bam*HI	37.9-39.8	(*Bgl*II)	SP1204
pVJ164	42.2-50.7	*Bgl*II	44.0-49.7	(*Eco*RI)	SP1205
pVJ438 [b]	4.8-8.0	*Cla*I	5.5	(*Bgl*II)	SP1252
pVJ431	2.8- 3.5	*Xba*I	3.1	(*Bgl*II)	SP1253
pDR5	5.8-10-4	*Eco*RI	8.3	(*Bgl*II)	SP1254
pDR2	18.7-20.6	*Eco*RI	19.5	(*Bgl*II)	SP1255
pLG130	33.0-37.2	*Xba*I	34.4	(*Xba*I)	SP1256
pLG152	25.2-27.4	*Xba*I/*Hin*dIII	26.3	(*Bam*HI)	SP1257
pDR18	26.9-28.6	*Hin*dIII	27.4	(*Bgl*II)	SP1258
pLG119 [c]	32.1-47.3	*Kpn*I	42.7	(*Bgl*II)	SP1259
pLG139 [d]	42.2-50.7	*Bgl*II	43.5	(*Xba*I)	SP1260

[a] Deleted fragments replaced with pVA891/the restriction site at which pVA891 inserted.

[b] Constructed by S. Al-Khaldi (1).

[c] pLG119 is a *Kpn*I plasmid from deletion mutant SP1203.

d pLG139 is a pUC8-derivative carries 2.3 kb *Eco*RI fusion fragment of pVJ164.

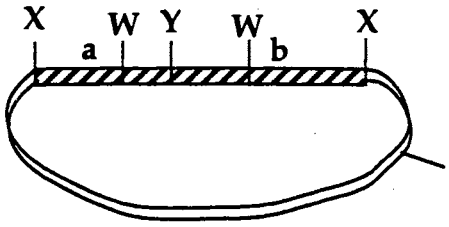
The newly created recombinant plasmids were digested with appropriate restriction enzymes, and a drug-resistance gene (pVA891 in this case) was ligated to either the linearized plasmid or a fragment of it. Upon ligation, the newly created circular molecule was digested with another enzyme that cleaved only at the vector portion but not at the drug-resistance gene. The linear molecule was used as donor DNA to transform SP1000 cells carrying Tn*5252* in order to introduce an insertion or deletion within the transposon. The method employed to insert a drug resistance marker heterologous to the recipient chromosome was possible due to flanking homology in the donor DNA for synapses and genetic integration into the recipient genome. The strategy is illustrated in Figure 10. This type of insertion of heterologous DNA is different from that of insertion-duplication mutagenesis in which circular donor DNA was used (76).

The strategy employed for deletion mutagenesis was essentially the same as for insertion mutagenesis. The cloned transposon DNA fragments on the recombinant plasmids were digested with a given restriction enzyme that recognizes more than one internal site in the passenger DNA. The internal restriction fragments were removed following restriction endonuclease digestion and replaced with the drug-resistance gene. The resulting circular chimeric molecule carrying a defined deletion was linearized with a restriction enzyme and introduced into SP1000 via transformation. The restriction enzyme was chosen in such a way that it could linearize the ligation product by providing a sufficient length of homology (more than 0.3 kb) flanking the drug-resistance gene.

The transformed SP1000 recipient cells were plated on CAT agar supplemented with 3 % blood as described previously. The Em^r transformants were screened for unselected markers, Cm^r and *str-1*, by

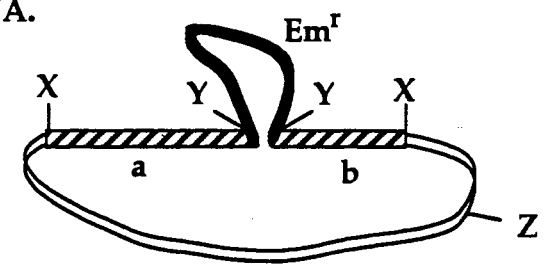
Figure 10. Strategy for creating insertion/deletion mutations within Tn*5252*. A DNA fragment derived from the transposon and flanked by the restriction sites, X, was cloned into an *E. coli* vector plasmid. The recombinant plasmid was linearized upon digestion with a restriction enzyme that cleaved at technique site, Y, and ligated to the drug-resistance gene (pVA891, Em^r) The resulting circle was again linearized at a site, Z, within the vector plasmid. The linear DNA was used as donor DNA to transform SP1000 cells carrying Tn*5252* and transformants were selected for erythromycin resistance. Panel D shows the expected insertion within Tn*5252*. Similar method was used for deletion mutagenesis. In this case, the passenger DNA in panel A was digested with another enzyme, W, all the internal fragments were removed and replaced with the marker gene.

A - Isolate and ligate a fragment from the transposon to a suitable vector

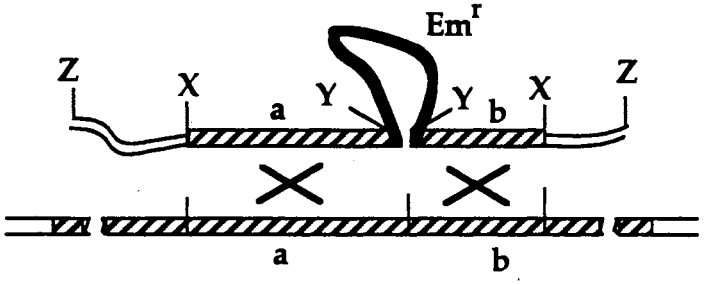


Recombinant plasmid

B - Insert a drug resistance marker into a unique restriction site in the passenger DNA.



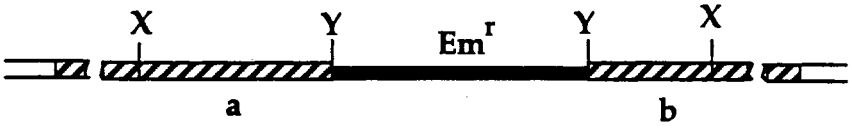
C - Linearize the vector at the restriction site Z, and use it to transform SP1000 (Tn5252)



donor DNA

Tn5252 in the chromosome

D - The resulting insertion mutant (Tn5252 ::Em^r)



replica plating. The putative mutants were analyzed by blot hybridization to confirm the result of the mutagenic events had taken place.

Introducing *in vivo* Deletions within Tn*5252* and Confirmation of Deletions by Southern Hybridization

The chromosomal DNAs from the putative deletion (replacement) mutants were prepared, digested with restriction enzymes, and separated on 0.8% agarose gels. The DNA samples were transferred to nylon membranes, and analyzed by Southern hybridization with appropriate probes to check whether the intended mutations had been created within the transposon. Of the six deletion mutants, two (deletion strains SP1200 and SP1201) were previously reported (1). The four newly generated deletion mutants are described below.

Deletion Strain SP1202

The *E. coli* plasmid pVJ162 containing the *tet* region from Tn*5253* was constructed by self-ligating 28.5 kb of a *Bgl*II fragment in which pVA891 had been inserted (77) . pVJ162 gave rise to pVJ162Δ*tet* after the spontaneous deletion of an 18-kb segment of DNA internal to the passenger fragment in *E. coli* in the absence of tetracycline selection (Vijayakumar, unpublished data). Therefore, the resulting deletion plasmid, pVJ162Δ*tet* (Figure 9), carried a 10.5-kb *Bgl*II fragment (coordinates 27.4 to 37.9). pVJ162Δ*tet* was digested with *Hin*dIII and a 3.0 kb fusion fragment carrying

the unique *Bgl*II site was extracted and ligated to *Hin*dIII digested pVA891. The recombinant plasmid, pLG121 (Figure 9), was generated in *E. coli*. This plasmid carries a 7.5-kb deletion (coordinates 28.6 to 36.1) internal to 10.5 *Bgl*II passenger fragment in pVJ162Δ*tet* (Table IX). In other words, the deleted internal region of passenger DNA in pVJ162Δ*tet* was replaced with pVA891. To delete the 7.5 kb *Hin*dIII fragments *in vivo* within the transposon, pLG121 was linearized at the unique *Bgl*II site, electrophoresed from a preparative gel to eliminate uncut plasmid molecules, and used as donor DNA to transform SP1000 cells carrying Tn*5252*. The erythromycin resistant-transformants were selected on CAT agar. To confirm the putative deletion mutants, a number of transformants were examined for their phenotypic and genotypic features by replica plating on selective CAT-agar plates.

Three of the putative deletion mutants were further analyzed by Southern hybridization to confirm the intended deletion. Chromosomal DNAs from the three transformants were prepared, digested with *Bam*HI, *Eco*RI and *Hin*dIII, separated by electrophoresis on an agarose gel, and transferred to nylon membrane. Chromosomal DNAs from Rx1 and SP1000, digested with the same enzymes, served as controls. The samples were probed with radiolabeled pVJ162Δ*tet* DNA. The membrane was washed and exposed to X-ray film. Autoradiogram is given in Figure 11. Lane a and lane b show Rx1 and SP1000 strains respectively, lanes c through e represent three putative deletion mutants, and lane m contains the molecular weight standards. As evident from the autoradiogram, Rx1 did not hybridize with either vector (pVA891) or insert portion of the probe. On the other hand, probe hybridized with the 6.9 kb, 4.2 kb and 3.3 kb *Bam*HI fragments, the 4.5 kb, 3.6 kb, 3.1 kb, 1.6 kb, and 0.9 kb *Hin*dIII fragments,

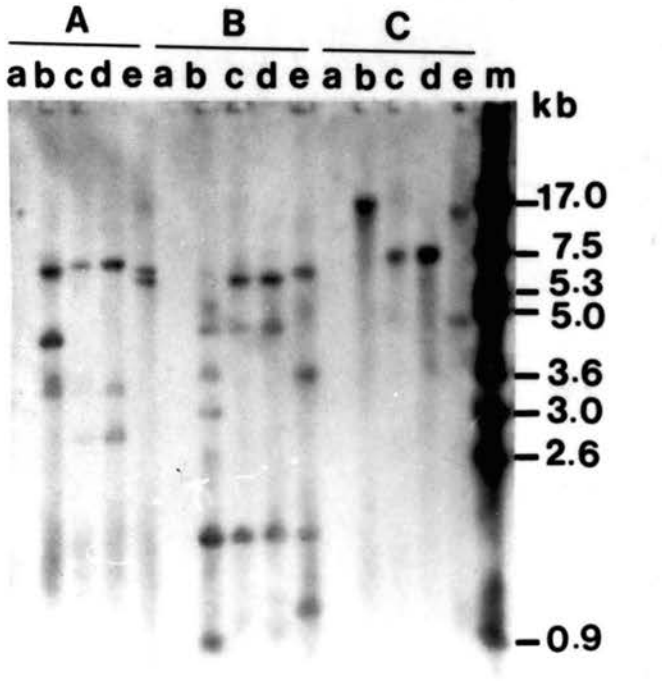


Figure 11. Autoradiogram Showing the Insertion of pVA891 within Tn*5252* on the Chromosome of SP1202. *Bam*HI (A), *Hin*dIII (B), and *Eco*RI (C) digested chromosomal DNAs were probed with ^{32}P-labeled pVJ162Δtet. a, Rx1; b, SP1000; c through e, putative deletion mutants; m, molecular weight marker. The clone in lane d was designated as SP1202.

and a 16.6-kb *Eco*RI fragment of SP1000. Deletions of specific fragments within the transposon and replacement of such fragments with the heterologous vector plasmid pVA891 resulted in the appearance of new fragments and the disappearance of others. Two of the putative deletion mutants (lane c and d) showed the same hybridization pattern for three restriction enzymes tested but the third one (lane e) had some chromosomal rearrangement.

The two deletion mutants gave the expected hybridization patterns. Three *Bam*HI fragments (6.6 kb, 3.3 kb, and 2.6 kb) arose as a result of deletion of 7.5 kb *Hin*dIII fragments (Table IX) and insertion of pVA891. Likewise, 3.6 kb, 3.1 kb, and 0.9 kb *Hin*dIII fragments were replaced by a 5.8-kb *Hin*dIII fragment which represented the vector plasmid pVA891 (Figure 8). A 16.6 kb *Eco*RI fragment from Tn*5252* gave rise to two fragments of 7.7 kb and 7.4 kb in the mutant strains as a result of pVA891 insertion. The strain yielding the sample in lane d was designated as SP1202. The physical map of strain SP1202 is illustrated in Figure 12.

Deletion Strain SP1203

The recombinant *E. coli* plasmid pVJ91 is flanked by *Kpn*I sites (coordinates 32.1 to 47.3) and derived from pneumococci carrying Tn*5253*. pVJ91, covering 15.2 kb DNA at the right end of the transposon (Figure 9), has three internal *Bam*HI fragments of 4.2 kb, 3.3 kb, and 1.1 kb in size (coordinates 33.0-41.6). The 10.5 kb *Bam*HI fusion fragment was purified and ligated to *Bam*HI cleaved pVA891. The product of ligation was linearized at the unique *Kpn*I site on the fusion fragment. The linearized

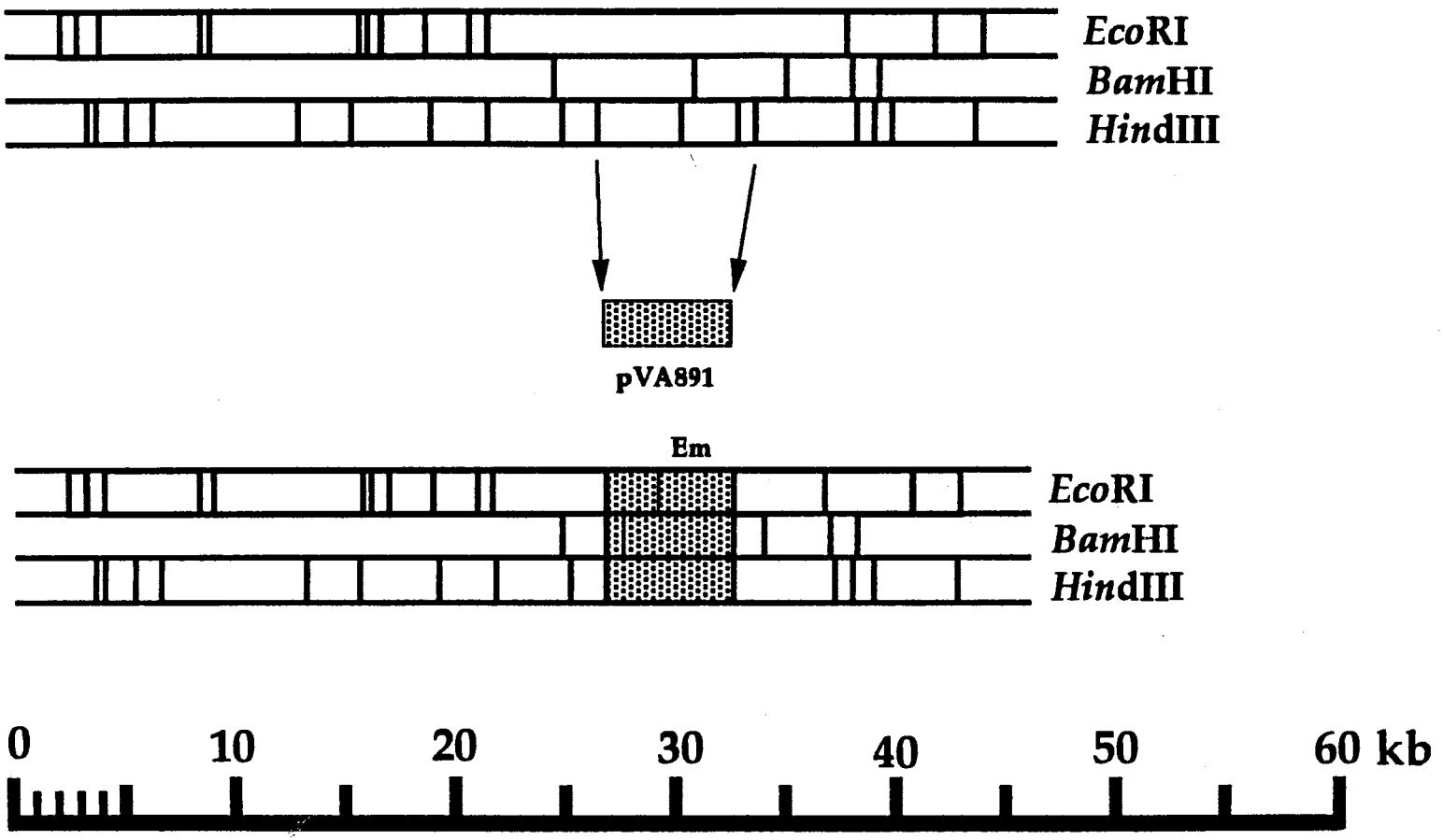


Figure 12. Physical Map of SP1202 Deletion Mutant and Orientation of pVA891 Insertion within Tn*5252*.

molecule was then used as donor DNA in transformation with SP1000 recipients as described previously. The Em^r transformants were confirmed by replica plating on selective CAT agar plates. After confirming the transformants, chromosomal DNAs from three putative deletion mutants were analyzed for intended deletions by blot hybridization.

The chromosomal DNAs were digested with *Bam*HI, *Hin*dIII and *Eco*RI and probed with pVJ91 DNA. The autoradiogram in Figure 13 shows the hybridization pattern of DNAs from the three transformants (lanes c, d, and e), Rx1 (lane a), and SP1000 (lane b). The *Bam*HI digest (pattern A) of SP1000 DNA showed five fragments of 9.0 kb, 6.7 kb, 4.2 kb, 3.3 kb, and 1.1 kb reacting with the probe. *Hin*dIII digest (pattern B) of SP1000 DNA showed eight fragments of 5.4 kb, 4.5 kb, 3.8 kb, 3.5 kb, 2.6 kb, 0.9 kb, 0.8 kb and 0.7 kb in size. Four *Eco*RI fragments (pattern C) of SP1000; 16.6 kb, 3.9 kb, 3.2 kb, and 2.2 kb in size also hybridized with the probe. As expected 4.2 kb, 3.3 kb, and 1.1 kb *Bam*HI fragments within the transposon in the mutant strains were deleted and replaced by pVA891 (pattern A, lane d) as indicated by the appearance of a 5.9-kb *Bam*HI fragment. The other *Bam*HI fragments, 9.0 kb and 6.7 kb, were common in both SP1000 and in the deletion strain as expected. Three of *Hin*dIII fragments, 4.5 kb, 0.8 kb, and 0.7 kb, disappeared as a result of deletion while two flanking fragments, 5.4 kb and 0.9 kb, increased in size to 6.2 kb and 1.0 kb respectively upon pVA891 insertion (pattern B, lane d). The 16.6 kb *Eco*RI fragment reduced down to 12.9 kb, and the 3.9 kb fragment increased in size to 4.7 kb (pattern C, lane d) because of deletion of transposon DNA and insertion of pVA891. The deletion strain in lane d was designated as SP1203 and its physical map is illustrated in Figure 14.

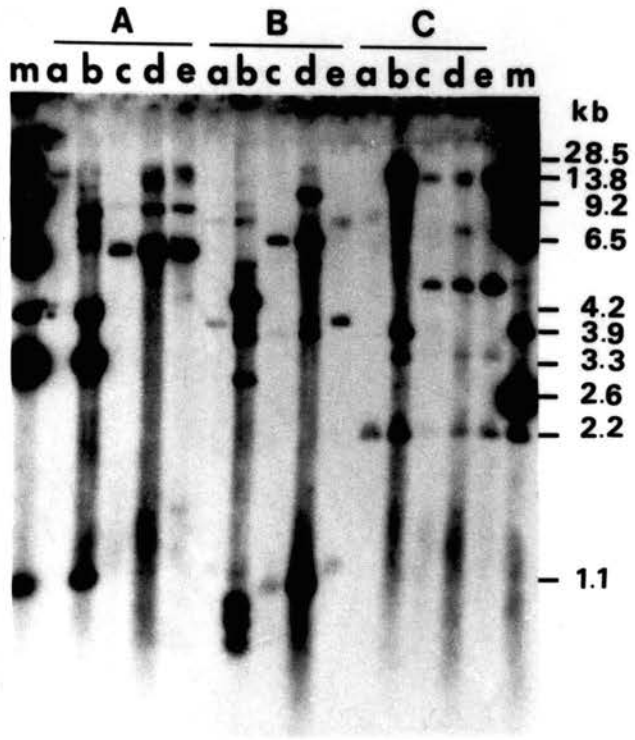


Figure 13. Autoradiogram Showing the Insertion of pVA891 within Tn*5252* on the Chromosome of SP1203. *Bam*HI (A), *Hin*dIII (B), and *Eco*RI (C) digested chromosomal DNAs were probed with ^{32}P-labeled pVJ91. a, Rx1; b, SP1000; c through e, putative deletion mutants; m, molecular weight marker. The clone in lane d was designated as SP1203.

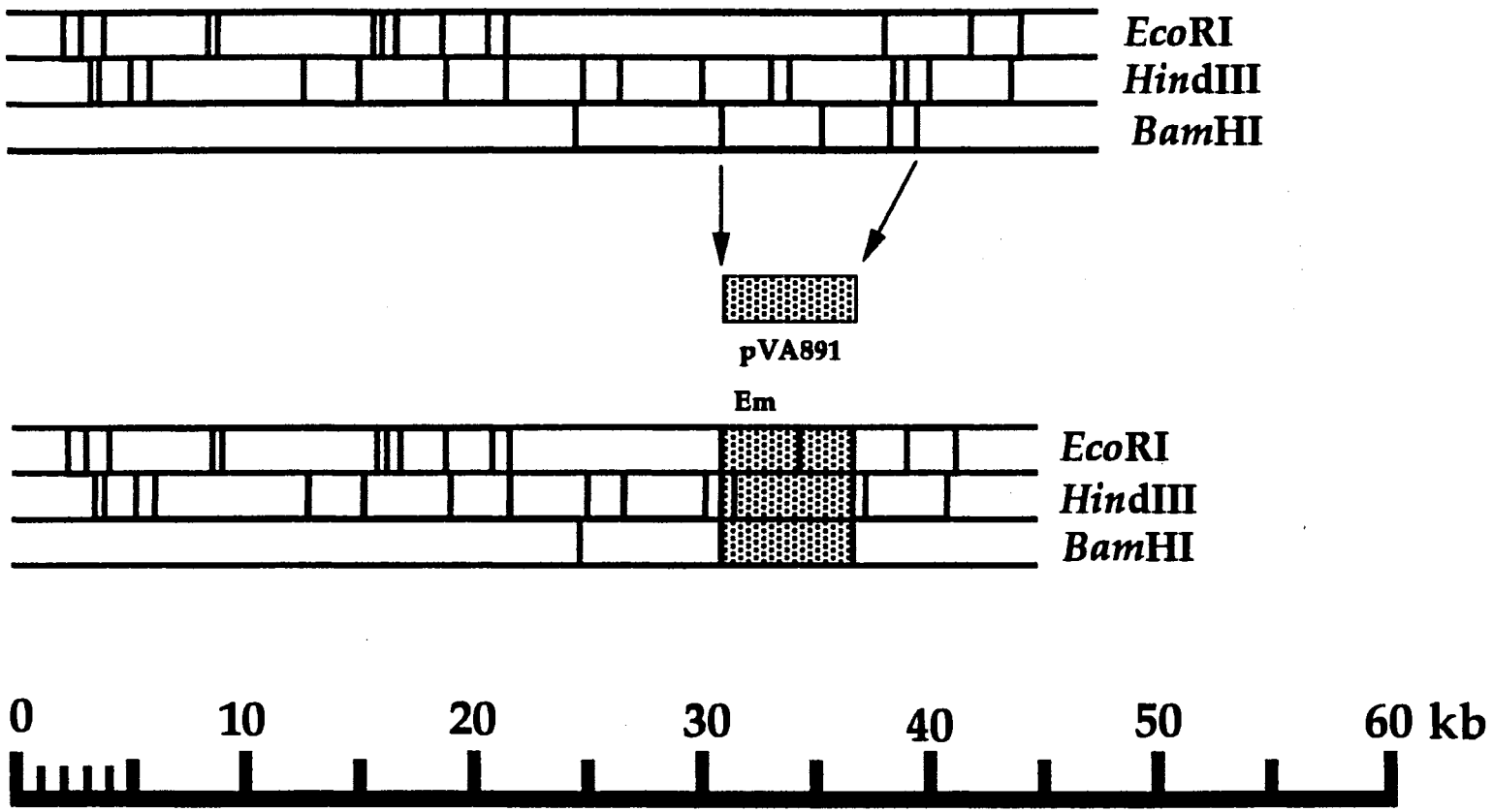


Figure 14. Physical Map of SP1203 Deletion Mutant and Orientation of pVA891 Insertion within Tn*5252*.

Deletion Strain SP1204

The 3.3 kb *Bam*HI fragment on the right end of Tn*5252* (coordinates 37.2 to 40.5) carried on pLG108 was used in the deletion of the 1.9-kb internal region flanked by *Bgl*II (coordinates 37.9 to 39.8) sites. pLG108 was digested with *Bgl*II and 1.9 kb *Bgl*II internal fragments removed and self ligated to generate the deletion derivative plasmid, pLG123 (Figure 9). pLG123 was digested at the unique *Bgl*II site and ligated to *Bam*HI digested pVA891. The circular chimeric plasmid was then linearized with *Bam*HI and used as donor DNA to transform the SP1000 recipient cells.

*Eco*RI and *Hin*dIII digests of chromosomal DNAs from Rx1, SP1000, and three putative deletion mutants were probed with ^{32}P-labeled pLG108. As evident from the autoradiogram (Figure 15), *Eco*RI- and *Hin*dIII-digested Rx1 DNA did not hybridize with the probe (lane a) whereas SP1000 showed two *Eco*RI fragments, 16.6 kb and 3.9 kb, as expected and some background fragments of varying sizes, and a single 4.5 kb *Hind*III fragment (pattern B, lane b). Three putative deletion mutants are shown in lanes c, d, and e. Of the three examined, one putative deletion mutant (lane c) gave the fragments expected upon the site-directed insertion of pVA891 within Tn*5252*. Three *Eco*RI fragments 16.9 kb, 3.9 kb, and 2.7 kb, and two *Hind*III fragments, 6.3 kb and 2.1 kb, hybridized with the probe. These were the expected results following the insertion of pVA891 insertion in one of the two possible orientations. The deletion mutant in lane c was designated as SP1204. The restriction endonuclease map of SP1204 showing the orientation of pVA891 is given in Figure 16.

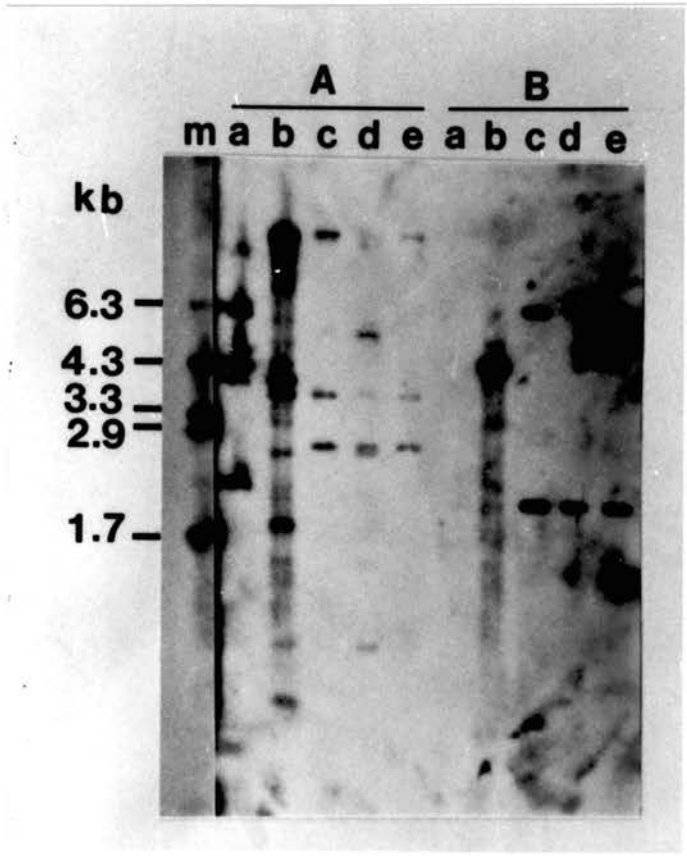


Figure 15. Autoradiogram Showing the Insertion of pVA891 within Tn*5252* on the Chromosome of SP1204. *Eco*RI (A) and *Hin*dIII (B) digested chromosomal DNAs were probed with ^{32}P-labeled pLG108. a, Rx1; b, SP1000; c through e, putative deletion mutants; m, molecular weight marker. The clone in lane d was designated as SP1204.

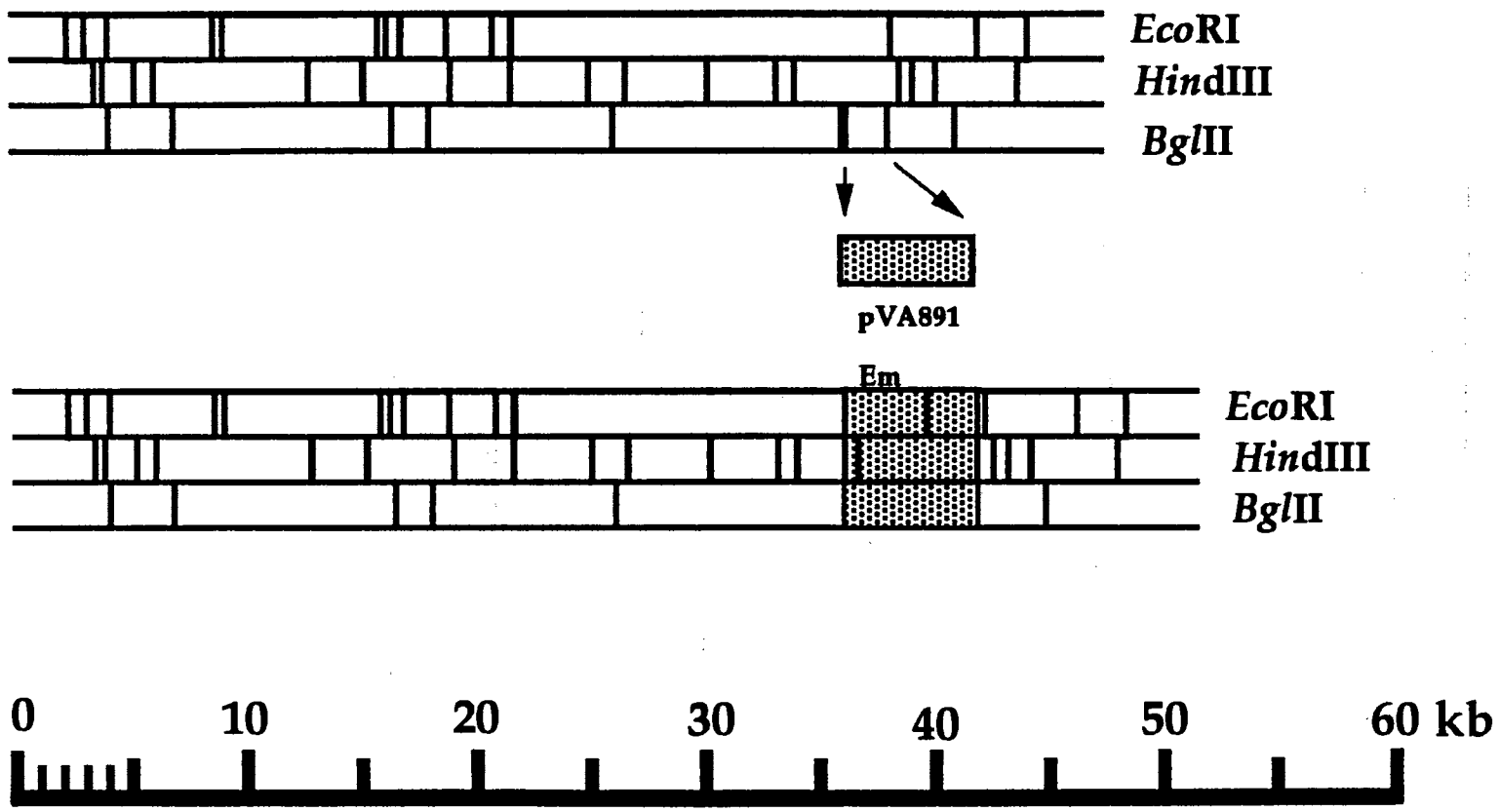


Figure 16. Physical Map of SP1204 Deletion Mutant and Orientation of pVA891 Insertion within Tn*5252*.

Deletion Strain SP1205

pVJ164 is a recombinant plasmid flanked by *Bgl*II sites and covering 7.9 kb fragment (coordinates 42.2 to 50.7) from the right junction of Tn*5252* (Figure 9). Of the 7.9 kb passenger fragment, about 1.0 kb is from outside of the transposon. A 3.2-kb *Eco*RI fragment covers the right terminus of the transposon (Figure 17). The 2.3 kb *Eco*RI fusion fragment of pVJ164 was extracted and ligated to the *Eco*RI cleaved pVA891. The circular molecule was linearized at the unique *Bgl*II site and used as donor DNA to transform SP1000 recipient cells. One of the Em^r transformants (lane c) was further analyzed for the deletion in blot hybridization experiments along with Rx1 (lane a), and SP1000 (lane b) and probed with a 3.9 kb *Xba*I fragment (coordinates 43.5 to 47.5) derived from the passenger DNA in pVJ164 (Figure 9 and Table VIII). Three *Eco*RI fragments; 3.9 kb, 3.2 kb, and 2.2 kb of SP1000 (pattern A, lane b) reacted with the probe and the 3.2 kb *Eco*RI fragment disappeared as expected (pattern A, lane c) from the mutant strain. It was not possible to decide, however, whether the second internal *Eco*RI fragment, 2.2 kb, disappeared because of the presence of a fragment of the same size in the Rx1 chromosome (pattern A, lane a). Homology between Rx1 chromosome and a portion the transposon DNA at its right terminus has been reported (77). The probe reacted with two fragments of 5.4 kb and 3.7 kb of *Hin*dIII-digested SP1000 DNA (pattern B, lane b). The putative deletion mutant reacted with a 4.3 kb *Hin*dIII fragment as expected (pattern B, lane c). The presence of the two unexpected *Hin*dIII fragments (approximately 3.7 kb and 3.2 kb) and a 5.5-kb *Eco*RI fragment might have been the result of partial digests of the chromosomal DNAs from the putative strain. These findings therefore indicated that the intended deletion

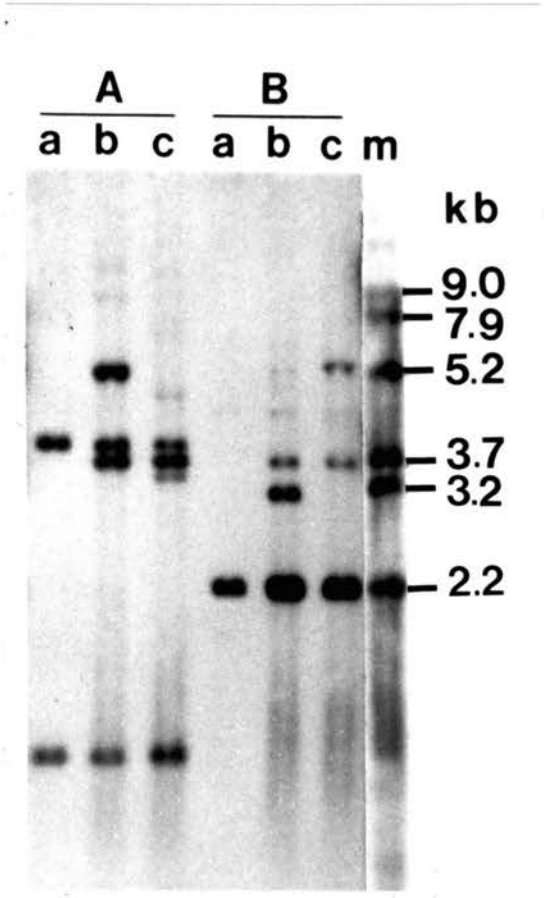


Figure 17. Autoradiogram Showing the Insertion of pVA891 within Tn*5252* on the Chromosome of SP1205. *Hin*dIII(A) and *Eco*RI (B) digested chromosomal DNAs were probed with ^{32}P-labeled pLG108. a, Rx1; b, SP1000; c, putative deletion mutant; m, molecular weight marker. The clone in lane c was designated as SP1205.

within the transposon took place as expected. Insertion of pVA891 within the transposon gave rise to deletion-mutant strain SP1205 (lane c). The physical map of SP1205 and the orientation of the pVA891 insertion within the transposon is given in Figure 18.

Insertion Mutagenesis of Tn*5252* and Physical Analysis of Insertions by Southern Hybridization

The *E. coli* plasmid vector pVA891 was used as a reporter gene to create insertions within Tn*5252*. However, the paucity of compatible restriction sites on both transposon DNA and on the marker gene rendered this method difficult to use extensively. Several attempts to subclone the transposon DNA fragments in *E. coli*, especially the ones at the right junction of the transposon, were not successful. Often, a portion of the passenger fragment was found to be deleted. Sometimes specific fragments were not clonable. Therefore, I resorted to creating insertion mutants to localize the transfer related regions. The basic strategy employed in this study is illustrated in Figure 10.

Insertion Strain SP1253

A 0.7 kb *Xba*I fragment (coordinates 2.8 to 3.5) at the left-most end of the transposon was carried on pVJ431 (Figure 9). This fragment did not carry any sites for the eleven restriction enzymes used to generate the restriction map of the transposon (Figure 7). However, during subculturing,

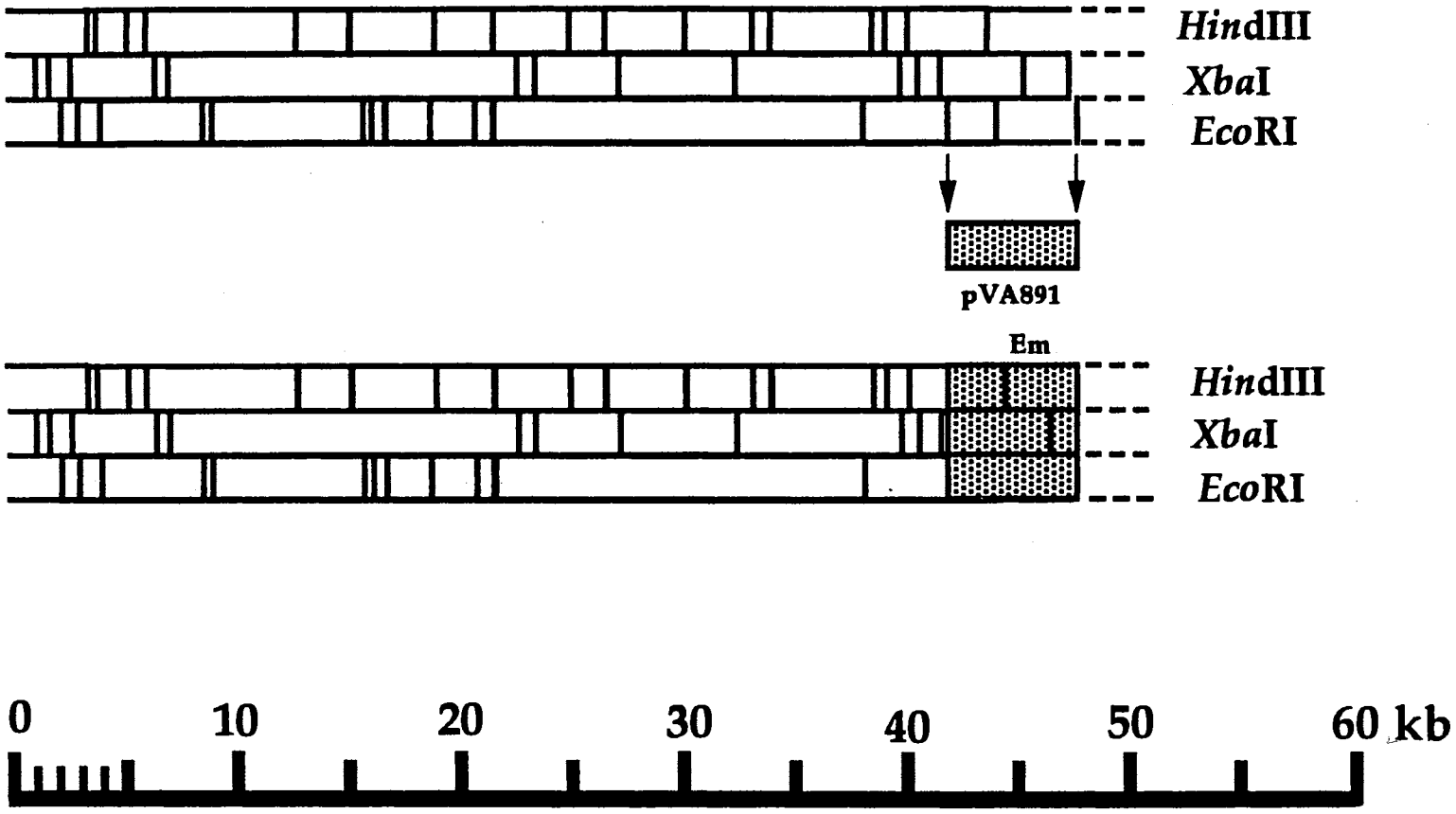


Figure 18. Physical Map of SP1205 Deletion Mutant and Orientation of pVA891 Insertion within Tn*5252*.

the recombinant plasmid had been modified and increased in size to about 1.2 kb. From DNA sequence analysis it was found that the IS*5* had transposed from *E. coli* chromosome into the middle of this fragment (Vijayakumar, unpublished data). The site of insertion of IS*5* into this fragment was 0.4 kb from the left end of the passenger fragment. The presence of a unique *Bgl*II site within IS*5* enabled me to insert the reporter gene within the 0.7 kb *Xba*I fragment and thus create an insertion within the transposon within this region.

*Bgl*II digested pVJ431 was ligated to *Bam*HI-digested pVA891, the ligation product was linearized at the *Kpn*I site on the vector portion of pVJ431, and then used as donor DNA in transformation of SP1000 recipient cells. Analyses of four Em^r transformants in blot hybridization confirmed that insertion of the reporter gene in the transposon was as expected and no other rearrangement was present (Figure 19). *Eco*RI-digested chromosomal DNAs from Rx1, SP1000, and four putative insertion mutants were probed with ^{32}P-labeled pSP110 carrying the 0.7 kb *Xba*I fragment (Figure 9, coordinates 2.8 to 3.5) . The radioactively labeled probe reacted with a 3.5-kb *Eco*RI fragment of the control strain, SP1000 (Figure 19, lane b) and it did not react with any *Eco*RI fragment of Rx1 (lane a) as expected. The probe reacted with two *Eco*RI fragments, 4.0 kb and 3.3 kb, of each insertion mutant (lanes c through f) as expected. In all four insertion mutants, pVA891 insertion within the transposon was in the same orientation (Figure 20). One of the deletion mutants (in lane c) was designated as SP1253 and used in further experiments.

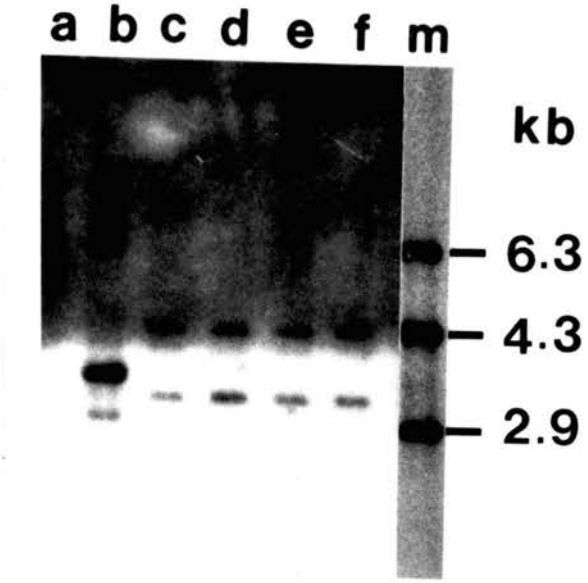


Figure 19. Autoradiogram of Southern Hybridization Showing the Insertion of pVA891 within Tn*5252* on the Chromosome of SP1253. *Eco*RI digested chromosomal DNAs were probed with ^{32}P-labeled pSP109. a, Rx1; b, SP1000; c through f, putative insertion mutants; m, molecular weight marker. The clone in lane c was designated as SP1253.

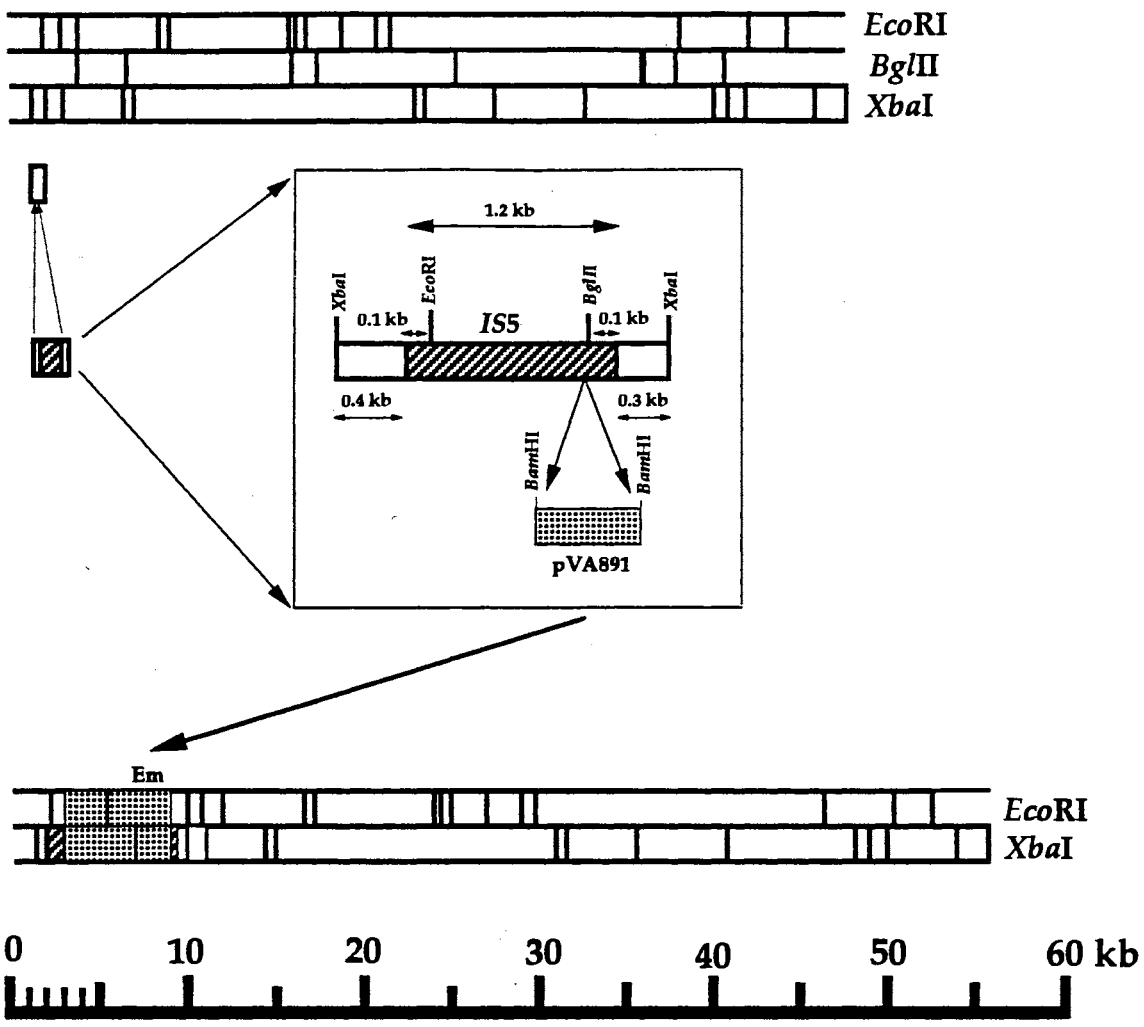


Figure 20. Physical Map of SP1253 Insertion Mutant. The diagonally striped area indicates IS*5* which inserted within 0.7 kb passenger DNA on the recombinant plasmid pVJ431. Insertion of the drug-resistance gene (pVA891), shaded box, into *Bgl*II site within IS*5* and its subsequent orientation within the transposon are shown. See text for details.

Insertion Strain SP1254

The recombinant plasmid, pDR5, carrying the 4.6 kb *Eco*RI fragment (coordinates 5.8 to 10.4) from the left end of the transposon (Figure 9) was cleaved at the unique *Bgl*II site within the insert and ligated to *Bam*HI digested pVA891 as described previously. The circular chimeric plasmid was linearized at the *Kpn*I site of the vector pDR5 following the ligation. Competent SP1000 cells were transformed with the linearized molecule to insert the reporter gene into the transposon. Of the 50 Em^r transformants, two were analyzed for the success of insertion mutagenesis by Southern hybridization. *Eco*RI- and *Hin*dIII-digested chromosomal DNAs were probed with ^{32}P-labeled pDR5 DNA.

The hybridization patterns of Rx1, SP1000, and the two putative mutant clones are illustrated in Figure 21. It is evident from the insertion of pVA891 into the transposon took place as expected. A 4.5-kb *Eco*RI fragment of SP1000 (pattern A, lane b) was split into two, 6.0 kb and 4.6 kb fragments, as a result of pVA891 insertion (pattern A, lane c and d). Also, four *Hin*dIII fragments, 6.8 kb, 1.3 kb, 0.9 kb, and 0.2, from the transposon (pattern B, lane b), gave rise to five fragments, 7.1 kb, 5.9 kb, 1.3 kb and 0.9 kb (pattern B, lane c and d) indicating the insertion of pVA891 within the transposon as expected. The intensity of the 5.9 kb *Hin*dIII fragment was less than the others because only a 0.2-kb portion of it derived from the transposon, the rest derived from pVA891. Since the probe reacted only with the 0.2 kb portion, the intensity of 5.9 kb *Hin*dIII fragment was expected to be less. A physical map of SP1254 strain is illustrated in Figure 22.

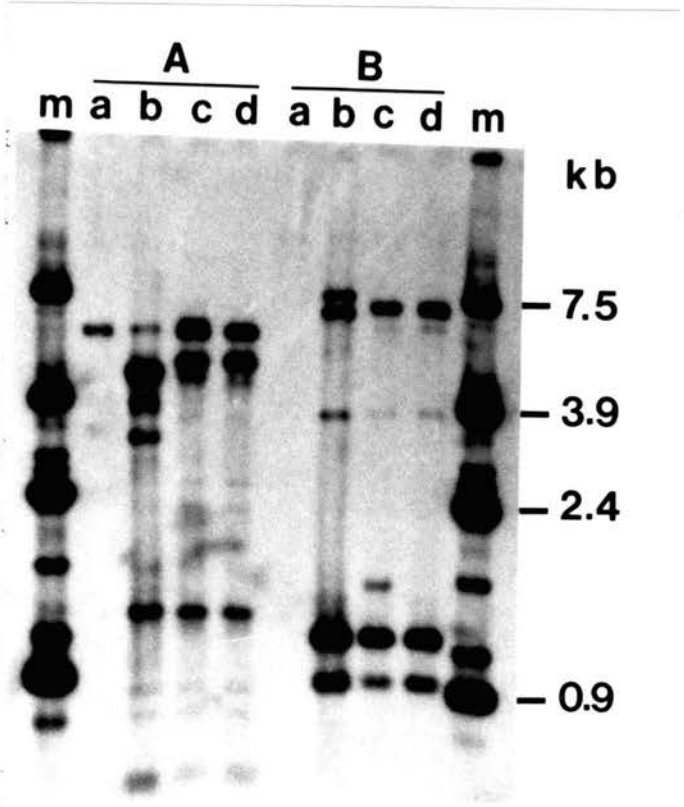


Figure 21. Autoradiogram of Southern Hybridization Showing the Insertion of pVA891 within Tn*5252* on the Chromosome of SP1254. *Eco*RI (A) and *Hin*dIII (B) digested chromosomal DNAs were probed with ^{32}P-labeled pDR5. a, Rx1; b, SP1000; c and d, putative insertion mutants; m, molecular weight marker. The clone in lane c was designated as SP1254.

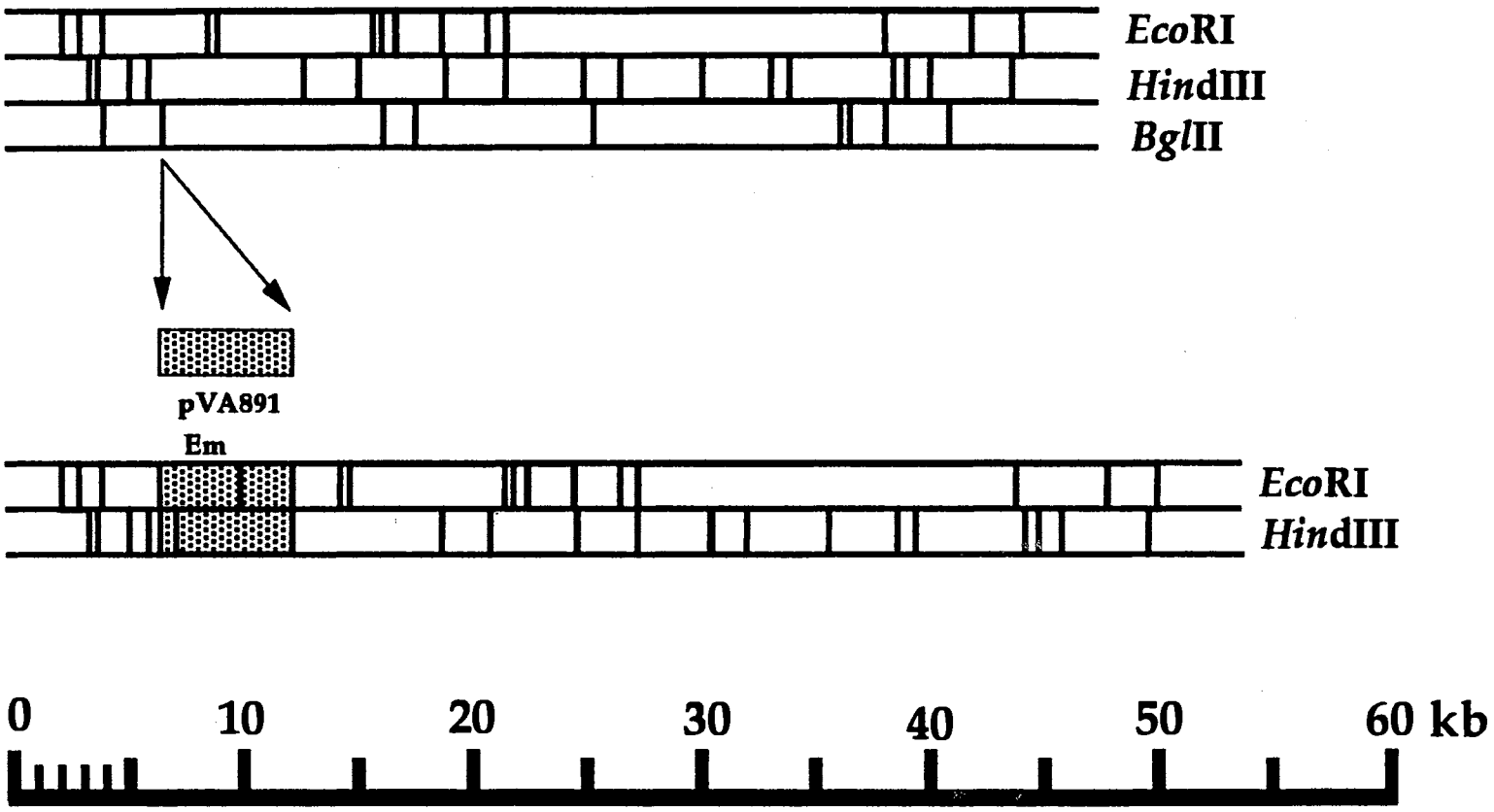


Figure 22. Physical Map of SP1254 Insertion Mutant and Orientation of pVA891 Insertion within Tn*5252*.

Insertion Strain SP1255

The 1.9 kb *Eco*RI fragment (coordinates 18.7 to 20.6) with a unique *Bgl*II site at the right end of the *cat* gene of the element was cloned on an *E. coli* plasmid to create the recombinant plasmid pDR2 (Figure 9). pDR2 was cleaved with *Bgl*II and ligated to pVA891 after *Bam*HI digestion. The construct was linearized with *Kpn*I at the vector portion of pDR2, and used as donor DNA to transform SP1000 competent cells. Two Em^r transformants were examined by Southern hybridization analyses for the directed insertion of the marker gene into the transposon. *Eco*RI- and *Hin*dIII-digested chromosomal DNAs from the putative insertion mutants along with Rx1 and SP1000 were probed with the radioactively labeled pDR2. As seen in the autoradiogram (Figure 23), the 1.9 kb *Eco*RI fragment of Tn*5252* (pattern A, lane b), gave rise to two *Eco*RI fragments in the insertion mutants (lanes c and d) as a result of pVA891 insertion. The probe hybridized with at least two *Eco*RI fragments in the Rx1 chromosome (pattern A, lane a). The background fragments present in the chromosomes of both SP1000 (lane b) and insertion mutants (lane c and d) may be due to partial restriction enzyme digestion. Depending on the orientation of the pVA891 insertion within the transposon, two *Eco*RI fragments, 4.3 kb and 3.4 kb, (lane c) or 4.5 kb and 3.1 kb, (lane d), were expected. Upon *Hin*dIII digestion, the 3.8 kb fragment (pattern B, lane b) of Tn*5252* was split into two fragments of 7.0 kb and 2.7 kb in size (pattern B, lane c) or of 7.8 kb and 1.8 kb (lane d) in the first mutant strain and the second mutant strain respectively. One of these mutants (lane c) was designated as SP1255 and further studied. The physical map of SP1255 is illustrated in Figure 24.

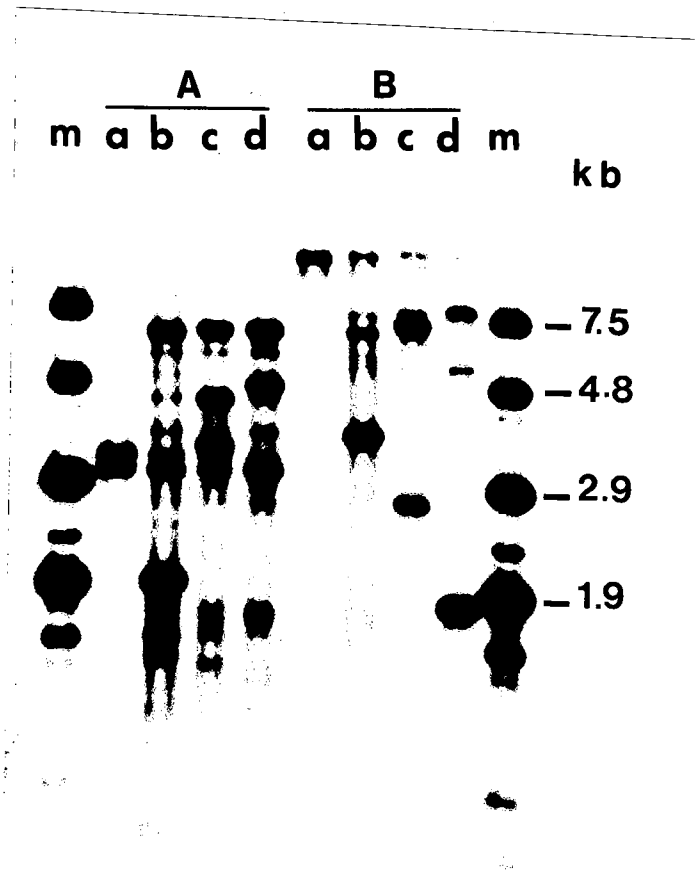


Figure 23. Autoradiogram of Southern Hybridization Showing the Insertion of pVA891 within Tn*5252* on the Chromosome of SP1255. *Eco*RI (A) and *Hin*dIII (B) digested chromosomal DNAs were probed with ^{32}P-labeled pDR2. a, Rx1; b, SP1000; c and d, putative insertion mutants; m, molecular weight marker. The clone in lane c was designated as SP1255.

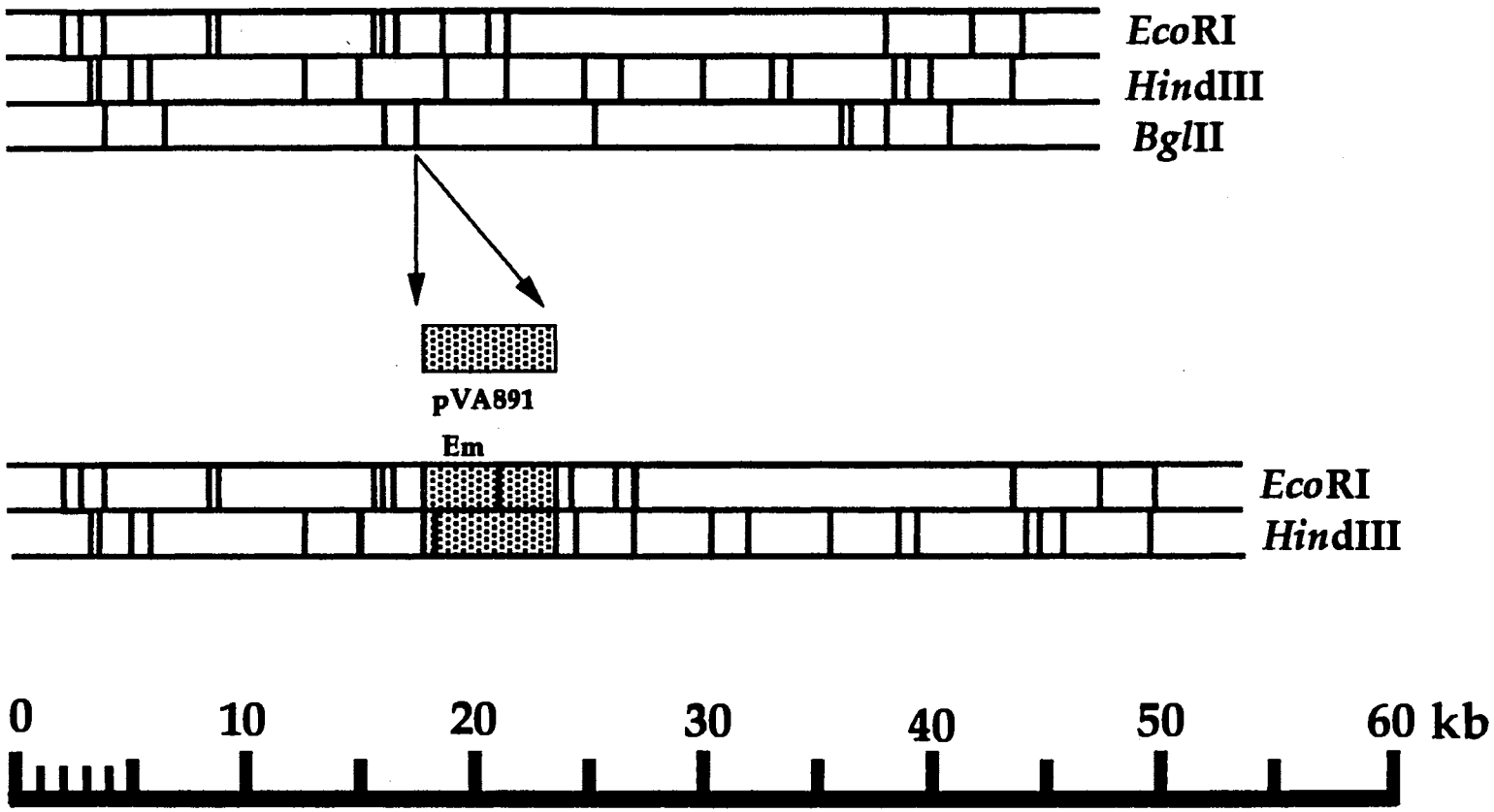


Figure 24. Physical Map of SP1255 Insertion Mutant and Orientation of pVA891 Insertion within Tn*5252*.

Insertion strain SP1256

The insertion mutant SP1256 was created as follows. A 4.2-kb *Bam*HI fragment (coordinates 33.0 to 37.2) was cloned into *Bam*HI digested *E. coli* plasmid, pUC8, to create the recombinant plasmid, pLG130 (Figure 9). This plasmid was digested with *Xba*I at the unique site on the passenger DNA fragment. The linear molecule was ligated to *Xba*I-cleaved pVA891. The ligation product was linearized with *Pst*I and used to transform SP1000 cells. The chromosomal DNAs isolated from two Em^r transformants were analyzed in blot hybridization using pLG130 (figure 9) as a probe. The probe reacted with a single 16.6 kb *Eco*RI (Figure 25, pattern A, lane b) and three *Hin*dIII fragments (pattern B, lane b) of sizes, 4.5 kb, 3.4 kb, and 0.9 kb of SP1000 parental cells. The probe was expected to react with 12.3 kb and 10.2 kb *Eco*RI fragments and 6.5 kb, 4.5 kb, 2.6 kb, and 0.9 kb *Hin*dIII fragments if pVA891 had inserted in one of the two orientations at this *Xba*I site. DNAs of the two putative mutants reacted with the probe as expected, confirming that the insertion was precise and no rearrangement took place due to the mutational event. Both putative insertion mutants had identical hybridization patterns indicating that pVA891 had inserted in one orientation. One of the mutant strains (in lane c) was named as SP1256, its physical map is illustrated in Figure 26.

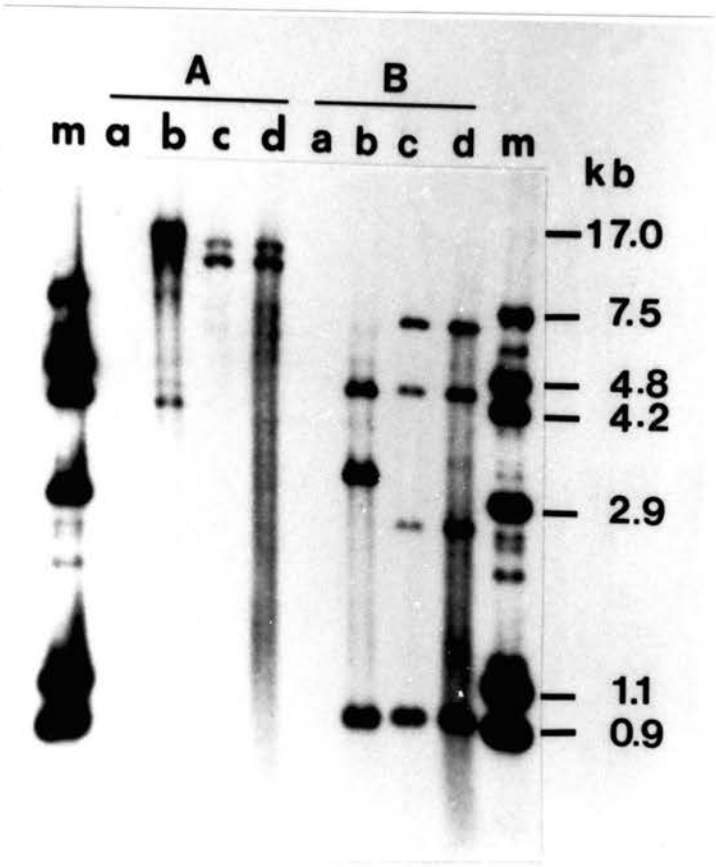


Figure 25. Autoradiogram of Southern Hybridization Showing the Insertion of pVA891 within Tn*5252* on the Chromosome of SP1256. *Eco*RI (A) and *Hin*dIII (B) digested chromosomal DNAs were probed with ^{32}P-labeled pLG130. a, Rx1; b, SP1000; c and d, putative insertion mutants; m, molecular weight marker. The clone in lane c was designated as SP1256.

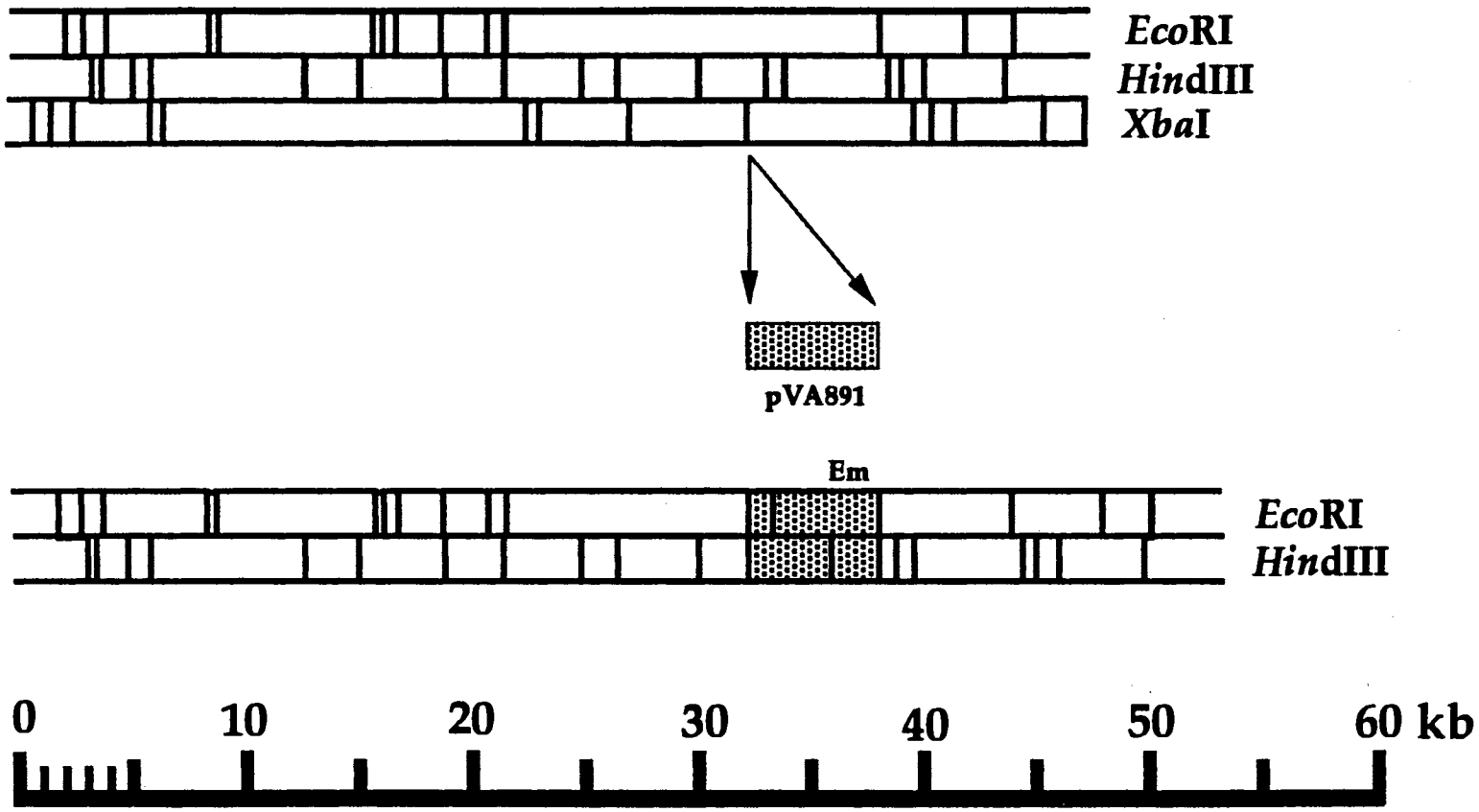


Figure 26. Physical Map of SP1256 Insertion Mutant and Orientation of pVA891 Insertion within Tn*5252*.

Insertion Strain SP1257

The recombinant plasmid pLG152 (Figure 9) was constructed by ligating a 1.8-kb *Xba*I/*Hind*III fragment from the middle region of Tn*5252* (coordinates 25.1 to 26.9) into an *Xba*I/*Hin*dIII-digested pBluescript SK+ vector. Since the *Bam*HI site in the multiple cloning sites of the vector plasmid was already removed by *Xba*I/*Hin*dIII double digest, the *Bam*HI site in the passenger DNA was used to insert the drug resistance gene. After ligating pVA891 and pLG152 at the *Bam*HI sites, the chimeric plasmid was cleaved at *Kpn*I site of the plasmid pLG152 and introduced into SP1000 recipients via transformation. Four Em^r transformants were tested by Southern hybridization for site-directed insertion of pVA891 within the transposon. Chromosomal DNAs were digested with *Eco*RI and *Hin*dIII and probed with pLG152. As evident from the autoradiogram (Figure 27), chromosomal DNA from Rx1 cells did not react with the probe (lane a). The radioactively-labelled probe reacted with a 16.6-kb *Eco*RI fragment from the transposon in the chromosome of SP1000 strain as expected (pattern A, lane b). The probe was expected to hybridize to two *Eco*RI fragments, 16.3 kb and 6.3 kb, of the insertion mutants. Two of the four putative insertion mutants (pattern A, lane d and f) reacted with the expected fragments in addition to at least four smaller fragments. A single *Hin*dIII fragment of 3.3 kb, from SP1000 (pattern B, lane b), was expected to react with the probe. In addition to this 3.3 kb fragment, two more *Hin*dIII fragments, more than 2 kb and 1 kb, from SP1000 strain hybridized with the probe. These extra fragments were believed to be due to star activity of the restriction enzymes. As it was evident from two clones (pattern B, lane d and f) that gave the same *Hin*dIII fragments,

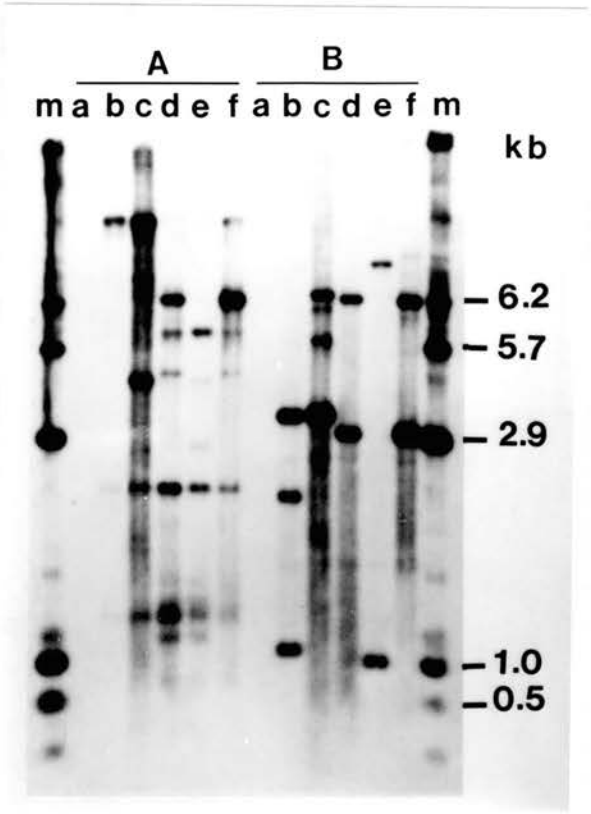


Figure 27. Autoradiogram of Southern Hybridization Showing the Insertion of pVA891 within Tn*5252* on the Chromosome of SP1257. *Eco*RI (A) and *Hin*dIII (B) digested chromosomal DNAs were probed with ^{32}P-labeled pLG152. a, Rx1; b, SP1000; c through f, putative insertion mutants; m, molecular weight marker. The clone in lane d was designated as SP1257.

6.2 kb and 3 kb, that the drug marker-gene was inserted within the transposon as intended without any chromosomal rearrangement, the insertion mutant shown in lane d was designated as SP1257 and chosen for further study. The physical map of SP1257 is given in Figure 28.

Insertion Strain SP1258

pDR18 carrying a 1.7-kb *Hin*dIII fragment with a unique *Bgl*II site in the middle region (coordinates 26.9 to 28.6) of Tn*5252* (Figure 9) was used to direct insertion of the reporter gene into the transposon. The *Bam*HI-digeted pVA891 was ligated to the *Bgl*II site within the passenger DNA. After linearizing in the vector portion with *Kpn*I, the ligation product was given to SP1000 recipients by transformation. To test for the insertion of pVA891 within the transposon, chromosomal DNAs from three Em^r transformants digested with *Eco*RI and *Hin*dIII were probed with radioactively labeled pDR18 in blot hybridization. Hybridization patterns of Rx1, SP1000, and three putative insertion strains are illustrated in Figure 29. A 16.6-kb *Eco*RI fragment from the transposon (pattern A, lane b) gave rise to two *Eco*RI fragments, 15.1 kb and 7.4 kb, as the result of pVA891 insertion within the transposon (lane c). The single 1.6 kb *Hin*dIII fragment from the transposon (pattern B, lane b) was also split into two fragments, 6.0 kb and 1.5 kb, due to pVA891 insertion as expected (pattern B, lane c). The physical map of this insertion mutant, SP1258, is illustrated in Figure 30.

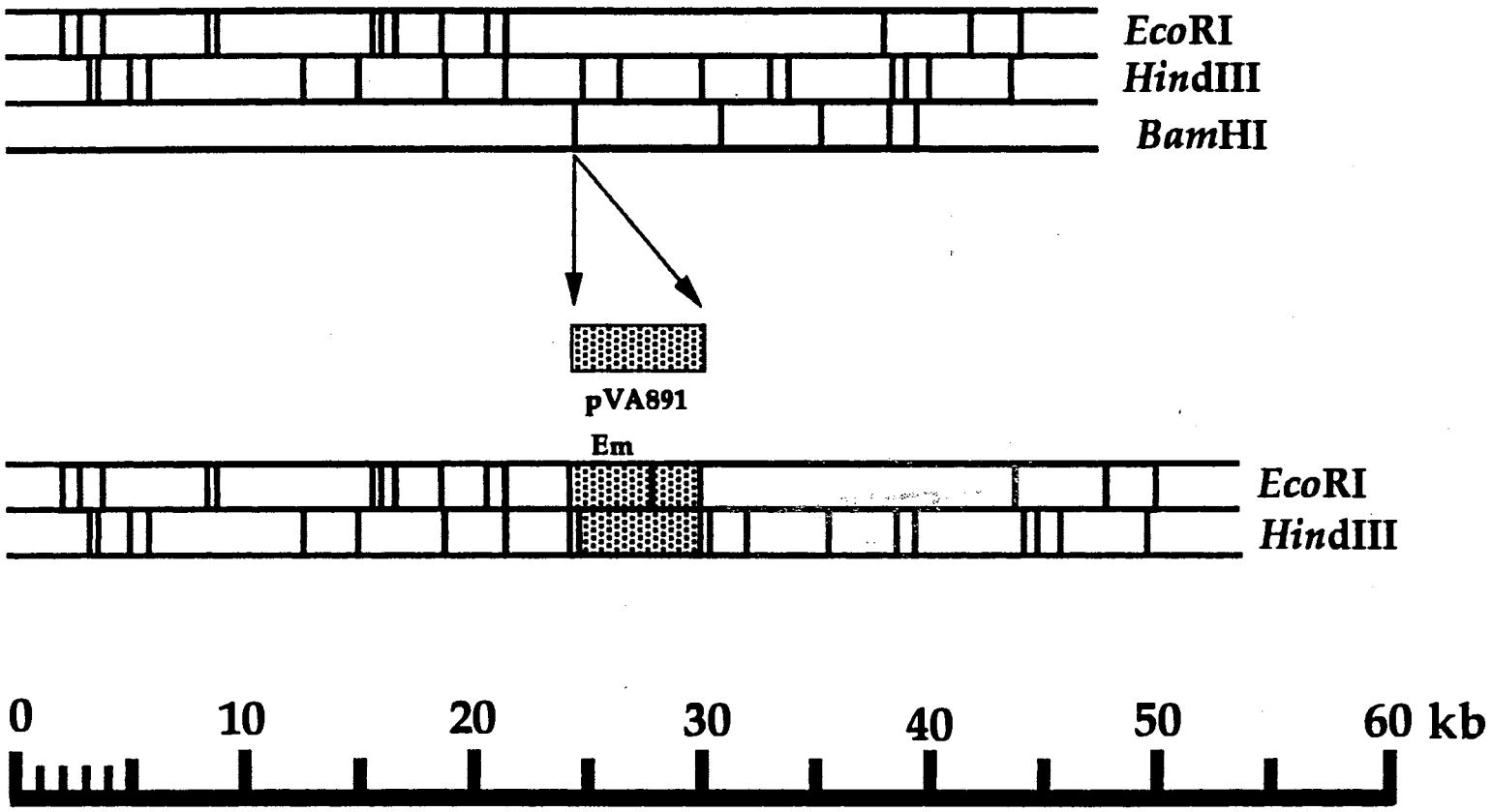


Figure 28. Physical Map of SP1257 Insertion Mutant and Orientation of pVA891 Insertion within Tn*5252*.

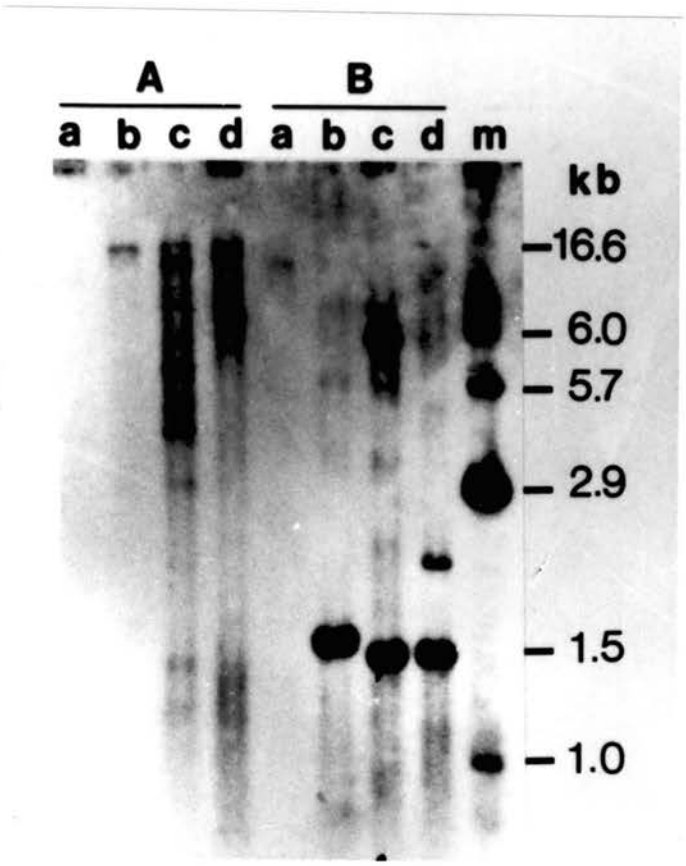


Figure 29. Autoradiogram of Southern Hybridization Showing the Insertion of pVA891 within Tn*5252* on the Chromosome of SP1258. *Eco*RI (A) and *Hin*dIII (B) digested chromosomal DNAs were probed with ^{32}P-labeled pDR18. a, Rx1; b, SP1000; c and d, putative insertion mutants; m, molecular weight marker. The clone in lane c was designated as SP1258.

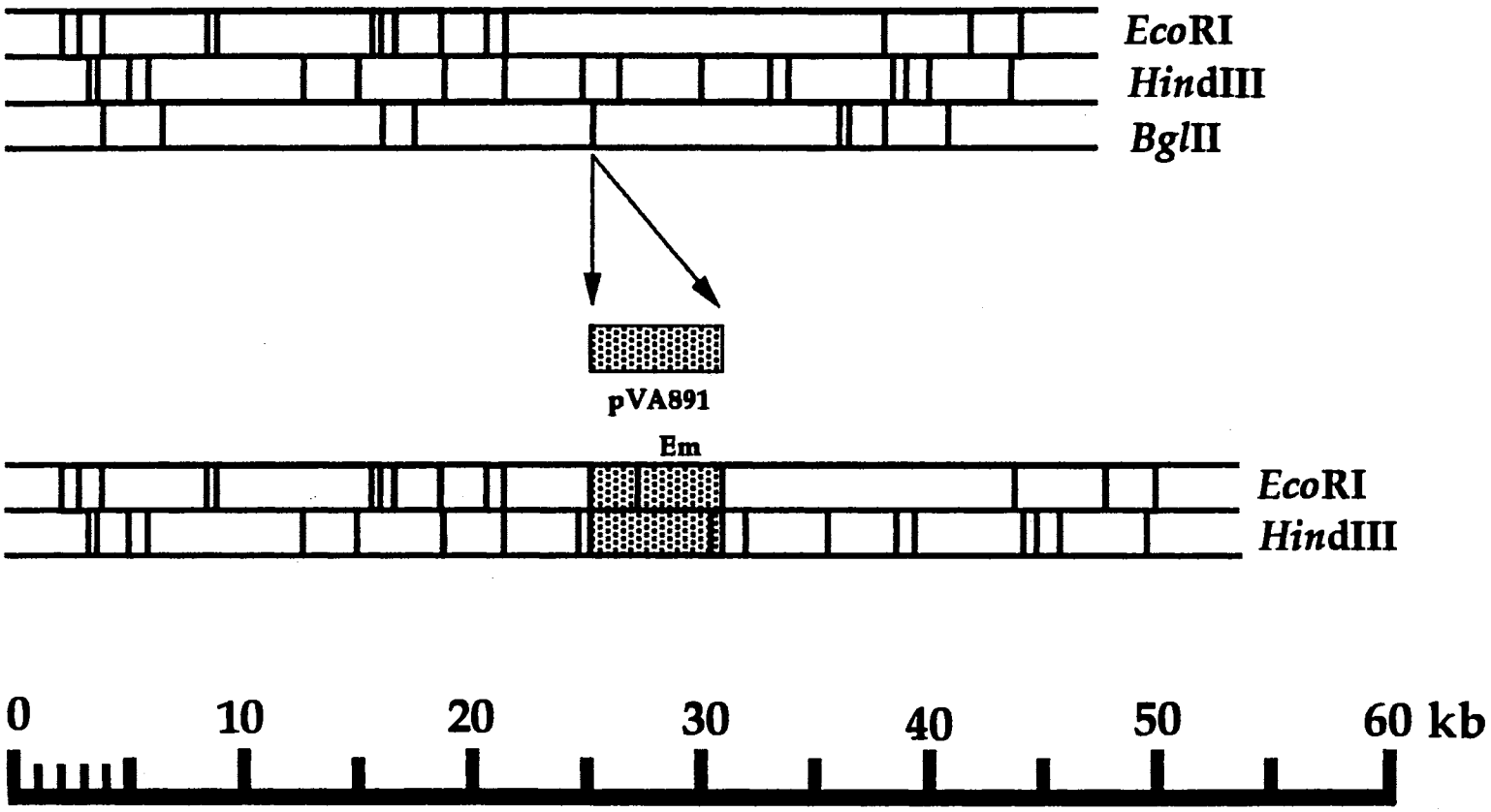


Figure 30. Physical Map of SP1258 Insertion Mutant and Orientation of pVA891 Insertion within Tn*5252*.

Insertion Strain SP1259

Chromosomal DNA from the deletion mutant, SP1203 (Figure 14), carrying pVA891, was digested with *Kpn*I, self ligated, and transformed into *E. coli* to generate the recombinant plasmid, pLG119 (figure 9). As described previously, the SP1203-deletion mutant was created by deleting 8.6 kb *Bam*HI fragments (coordinates 33.0 to 41.6) internal to a *Kpn*I fragment (coordinates 32.1 to 47.3, as in pVJ91), and replacing them with pVA891 (Table IX). The deletion-derivative plasmid pLG119 now carries a 0.9-kb *Kpn*I/*Bam*HI fragment at coordinates 33.0 to 32.1 and a 5.7-kb *Bam*HI/*Kpn*I fragment at coordinates 41.6 to 47.3 from the transposon. pLG119 was digested at the unique *Bgl*II site (coordinate 42.7) present in the passenger portion of pLG119. The linearized molecule was then ligated to *Bam*HI-cleaved pVA891. The ligation product was linearized with *Kpn*I and used as donor DNA to transform SP1000 cells. The chromosomal DNAs isolated from three Em^r transformants were analyzed by Southern hybridization using the 3.9-kb *Eco*RI fragment (coordinates 40.2 to 44.0) as a probe. The probe hybridized with one 3.9 kb *Eco*RI fragment (Figure 31, pattern A, lane b) and four *Hin*dIII fragments, 4.5 kb, 3.9 kb, 0.9 kb, and 0.7 kb in size (pattern B, lane b), of SP1000 cells. Upon the insertion of pVA891 at the *Bgl*II site, two *Eco*RI fragments, 6.0 kb, 3.8 kb (pattern A, lanes c through e), and five *Hin*dIII fragments, 9.0 kb, 4.5 kb, 0.9 kb, and two 0.7-kb (pattern B, lanes c through e) were expected to react with the probe. As evident from the autoradiogram, in all three mutants pVA891 insertion took place in one orientation . The presence of two *Eco*RI fragments with the expected sizes indicated that pVA891 insertion was precise and no rearrangement took place. Although three *Hind*III

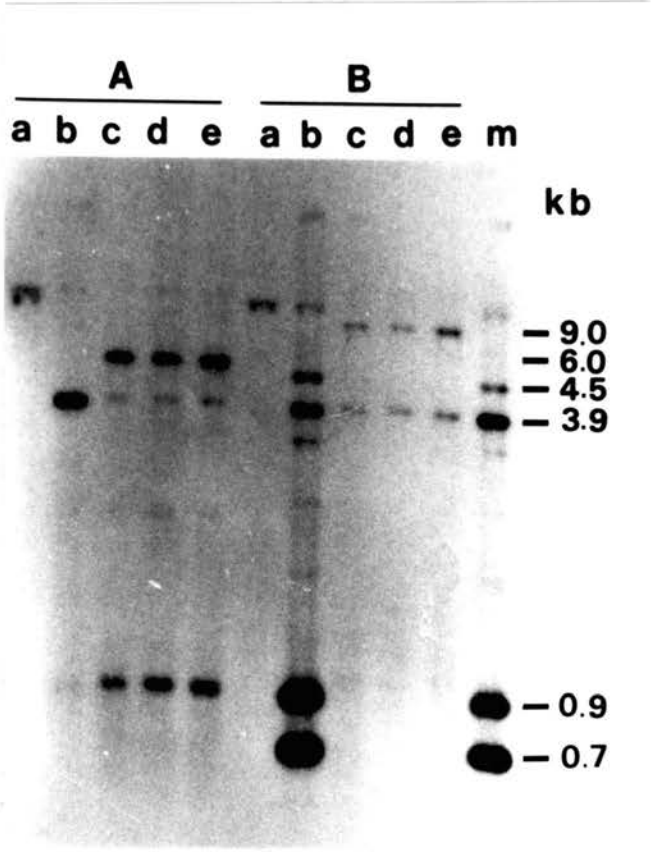


Figure 31. Autoradiogram of Southern Hybridization Showing the Insertion of pVA891 within Tn*5252* on the Chromosome of SP1259. *Eco*RI (A) and *Hin*dIII (B) digested chromosomal DNAs were probed with ^{32}P-labeled 3.9 kb *Eco*RI fragment (coordinates: 40.2-44.0). a, Rx1; b, SP1000; c through e, putative insertion mutants; m, molecular weight marker. The clone in lane c was designated as SP1259.

fragments (0.9 kb and two 0.7-kb) of the putative strains were not visible in the autoradiogram, the presence of two other *Hin*dIII fragments as expected confirmed that pVA891 was inserted at the *Bgl*II site in this region. The insertion mutant in lane c was designated as SP1259, and its physical map is given in Figure 32.

Insertion Strain SP1260

The 2.2 kb *Eco*RI fusion fragment from the recombinant plasmid pVJ164 was cloned into *Eco*RI-digested pUC8, to create pLG139 (Figure 9). This fusion fragment carries a 1.3-kb *Bgl*II/*Eco*RI fragment from coordinates 42.7 to 44.0 and a 0.9-kb *Eco*RI/*Bgl*II fragment from coordinates 49.7 to 50.6 (Table VIII). pLG139 was cleaved at the unique *Xba*I site (coordinate 43.5) and ligated to *Xba*I cleaved pVA891. The circular molecule was linearized with *Pst*I and used as donor DNA to transform SP1000 parental cells. Insertion of pVA891 into the *Xba*I site in this region was confirmed in two Em^r transformants by Southern hybridization. The probe, a 3.9-kb *Eco*RI fragment (coordinates 40.2 to 44.0), reacted with a 3.9-kb *Eco*RI (Figure 33, pattern A, lane b), and 4.5 kb, 3.9 kb, 0.9 kb, and 0.7 kb *Hin*dIII fragments (pattern B, lane b) of parental SP1000 strain. The orientation of pVA891 insertion in each mutant was found to be reversed. In one orientation, for example, as in one insertion mutant (lane c), two *Eco*RI fragments, 5.0 kb and, 4.8 kb, and five *Hin*dIII fragments, 5.3 kb, 4.5 kb (as a doublet), 0.9 kb and 0.7 kb, were expected to react with the probe. If the orientation of pVA891 was reversed, the probe was expected to hybridize with 7.9 kb and 1.8 kb, and 6.7 kb, 4.5 kb, 3.1 kb, 0.9 kb, and 0.7 kb *Eco*RI

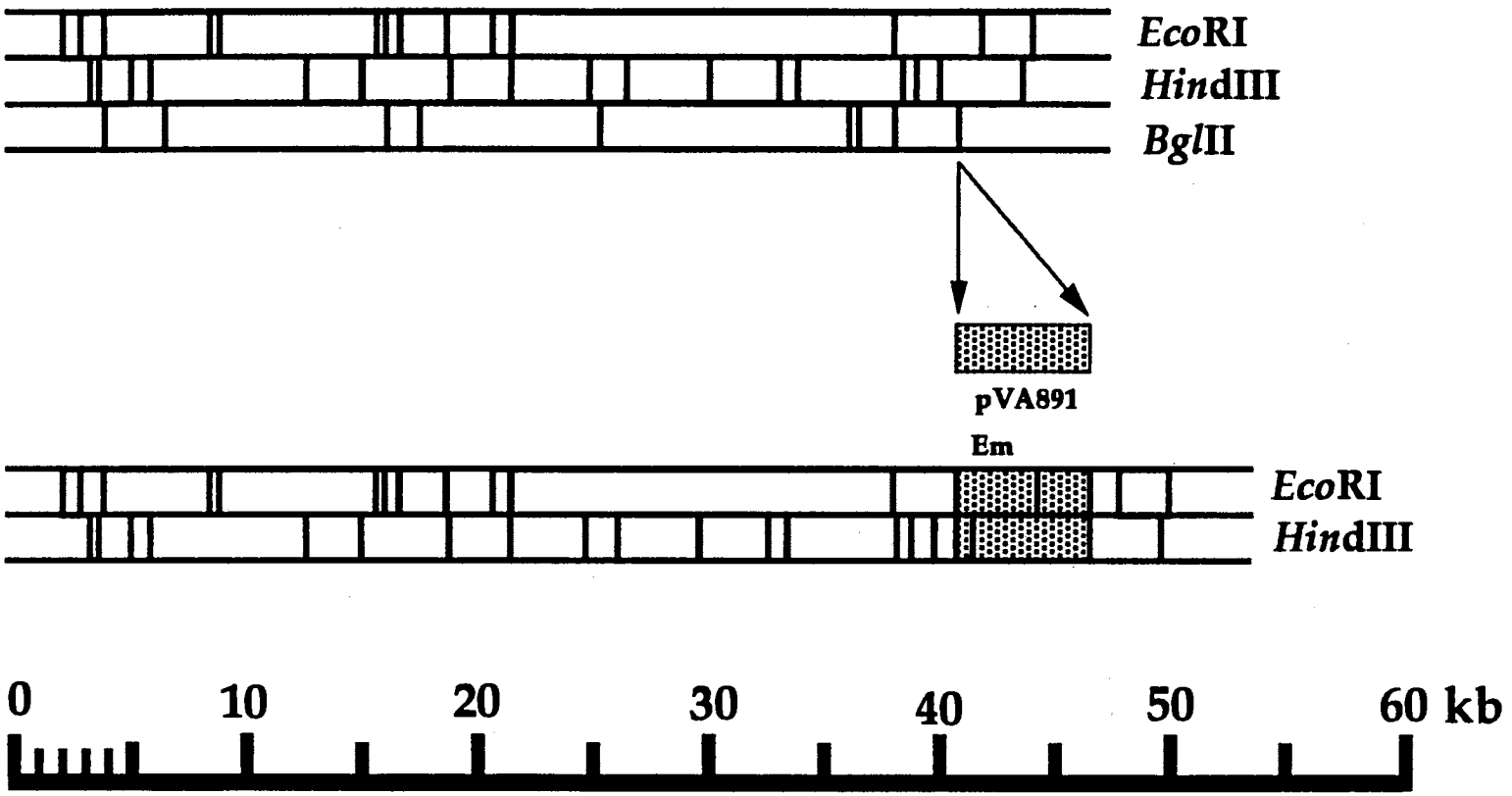


Figure 32. Physical Map of SP1259 Insertion Mutant and Orientation of pVA891 Insertion within Tn*5252*.

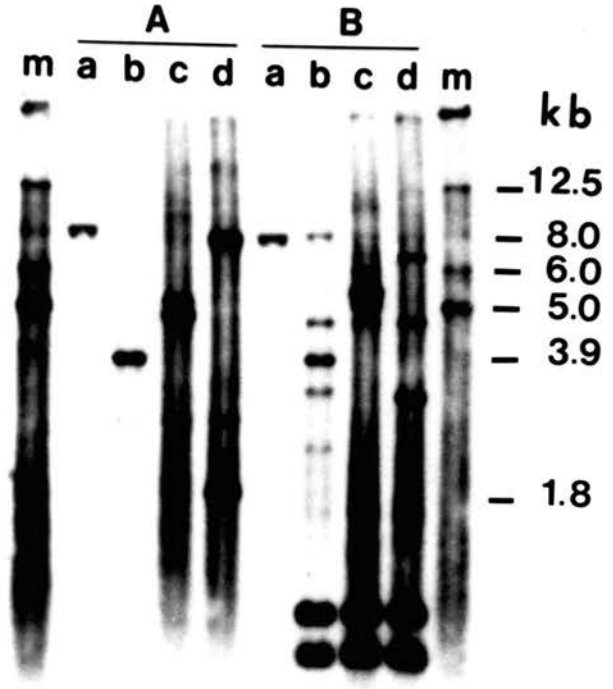


Figure 33. Autoradiogram of Southern Hybridization Showing the Insertion of pVA891 within Tn*5252* on the Chromosome of SP1260. *Eco*RI (A) and *Hin*dIII (B) digested chromosomal DNAs were probed with ^{32}P-labeled 3.9 kb *Eco*RI fragment (coordinates: 40.2-44.0). a, Rx1; b, SP1000; c through e, putative insertion mutants; m, molecular weight marker. The clone in lane c was designated as SP1260.

and *Hin*dIII fragments respectively (lane d). One of the insertion mutants (lane d) was designated as SP1260 and was used in further studies. The restriction map of SP1260 is given in Figure 34.

Conjugal Transfer Properties of Tn*5252* Deletion Mutants

The pneumococcal strains carrying specific deletions and insertions within Tn*5252* were tested for their ability to conjugally transfer from pneumococci to *S. pyogenes* recipient. Both pneumococcal donor strains and *S. pyogenes* recipient cells were cultured in CAT broth. The cells were mated on a filter for 4 h as described previously.

After confirming the deletions within Tn*5252* by Southern hybridization, each deletion mutant was examined for its conjugal-transfer properties in filter mating experiments with *S. pyogenes* recipients. The filter matings between deletion mutants and *S. pyogenes* were performed as described previously except that two-to-three fold more donor and recipient cells were mixed and mated on separate filters as replicates to detect transconjugants of low frequency. After mating, the filters were washed separately, the filter washes were pooled together, and plated on selective agar plates to score transconjugants. The transconjugants were selected on CAT agar containing optochin (recipient marker) and erythromycin (donor marker). The Em^r transconjugants were screened for unselected markers, chloramphenicol (transposon marker), and streptomycin (chromosomal marker of donor strains) by replica plating. Concentrations of selective drugs used for different bacteria are listed in Table III.

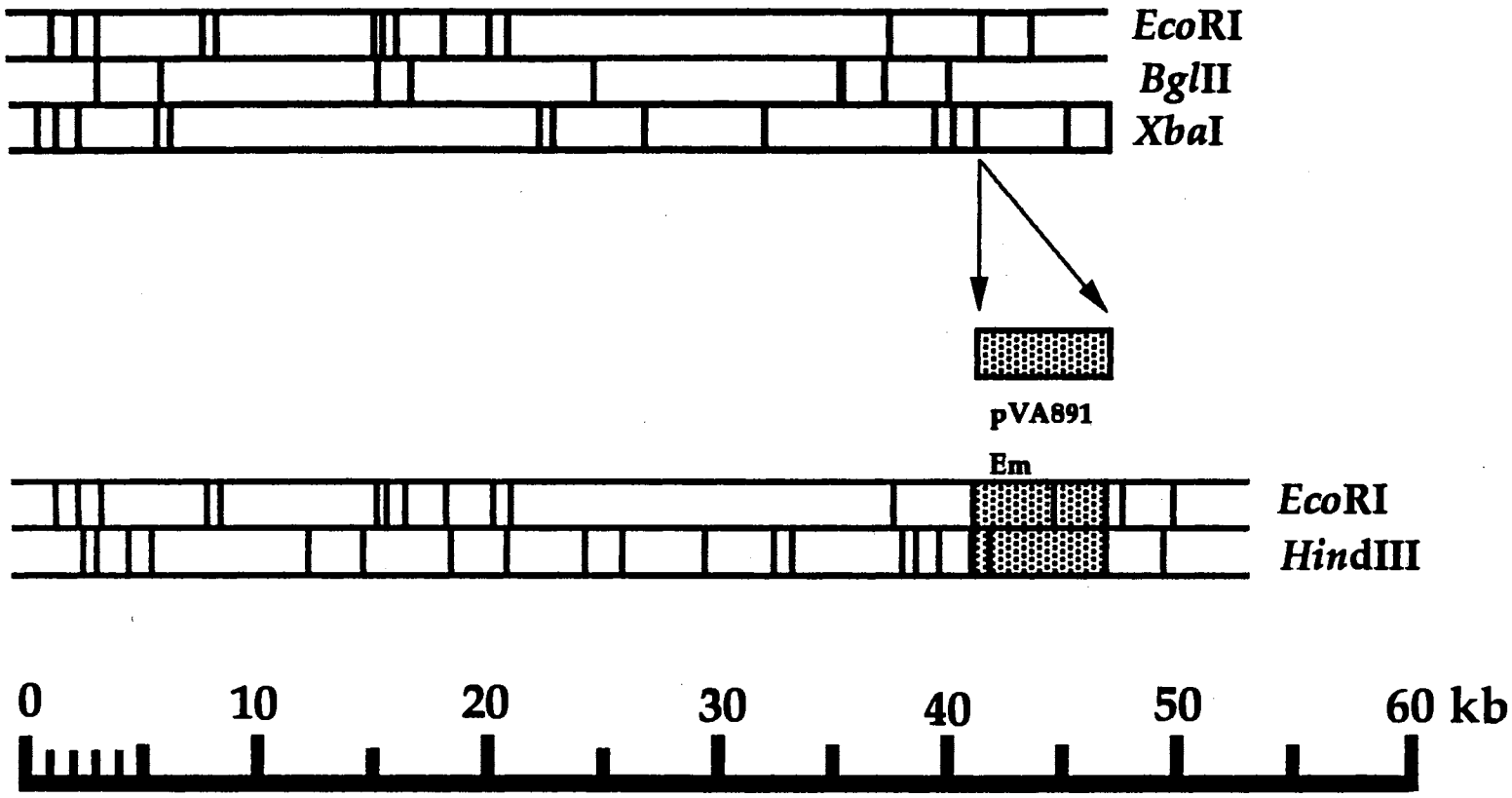


Figure 34. Physical Map of SP1260 Insertion Mutant and Orientation of pVA891 Insertion within Tn*5252*.

None of the six deletion mutants of Tn*5252* tested in filter matings were able to transfer to *S. pyogenes* recipients at a detectable frequency as compared to the control strain GP42 (Table X). The conjugal transfer frequencies of deletion mutants obtained from at least three different filter mating experiments were found to be less than 10^{-9} to 10^{-8} per donor cell whereas GP42, a transfer proficient Tn*5253* strain, gave10^{-6} transconjugants per donor cell. Effects of deletion mutations on the conjugal transfer frequency of Tn*5252* are summarized in Table X. These results indicated that transfer-related genes of Tn*5252* may be scattered throughout the element.

Conjugal Transfer Properties of Tn*5252* Insertion Mutants

Of the nine insertion mutants, five did not affect the conjugal transfer of Tn*5252*. Conjugal-transfer frequencies of these transfer-proficient insertion mutants were found to be 10^{-7} to 10^{-6} per donor cell (Table X).

Differences in ability of each mutant to transfer Tn*5252* via conjugation is considered to be insignificant. It is, however, possible that insertion of pVA891 in the vicinity of a functional gene might have interfered with the efficient expression of such a gene. For example, if the pVA891 had inserted in the immediate upstream region or downstream of a gene it might have altered the rate of its transcription.

These data indicate that the transfer-related genes are clustered at one of the termini, if not both, at the left of the element. Insertions in the middle of the element did not have any effect on the excision, intracellular transfer or integration of Tn*5252* in the recipient chromosome. The observation that

TABLE X

EFFECTS OF DELETION/INSERTION MUTATIONS ON THE CONJUGAL TRANSFER FREQUENCY OF Tn*5252*

Donor	Em^r/Cm^r Transconjugants/donor
SP1200	$< 2.0 \times 10^{-9}$
SP1201	$< 1.7 \times 10^{-9}$
SP1202	$< 2.5 \times 10^{-9}$
SP1203	$< 6.0 \times 10^{-9}$
SP1204	$< 2.3 \times 10^{-9}$
SP1205	$< 1.4 \times 10^{-9}$
SP1252	$< 2.2 \times 10^{-9}$
SP1253	$< 4.5 \times 10^{-9}$
SP1254	6.8×10^{-6}
SP1255	2.6×10^{-7}
SP1256	$< 3.8 \times 10^{-9}$
SP1257	2.1×10^{-7}
SP1258	3.5×10^{-7}
SP1259	$< 3.1 \times 10^{-9}$
SP1260	6.4×10^{-6}
GP42	1.3×10^{-6}

Each entry is the average of numbers obtained at least three independent matings.
Recipient was *S. pyogenes* ATCC 21547.
GP42 was used as control (Table I).

heterologous elements such as Tn*5251* and *cat* often are found inserted in the middle of large transposons reinforces these conclusions.

Confirmation of pVA891 Insertions within Tn*5252* by Transformation

All six mutants carrying deletions and four of nine insertion mutants were found to be deficient in conjugal transfer from pneumococcal donors to *S. pyogenes* recipients. However, five of the nine insertion mutants were able to transfer to *S. pyogenes* at a frequency comparable to that of the parental strain (Table X). In order to confirm the insertion of pVA891 within the transposon, *S. pyogenes* transconjugants were selected separately with erythromycin as well as chloramphenicol. The transconjugants were then screened for the unselected markers by replica plating on CAT agar. One of the Em^r and Cm^r transconjugants of *S. pyogenes* was further confirmed in transformation experiments. Chromosomal DNAs from the five *S. pyogenes* transconjugants which demonstrated transfer-proficient-Tn*5252* insertion mutants, SY152, SY153, SY154, SY158 and SY159 (Table I) were used as donor DNA to transform Rx1 and DP1333 recipient cells. Since the homology between Tn*5252* and the *S. pyogenes* chromosome was expected to be very limited, Em^r and Cm^r transformants would be expected to arise due to the homology provided by the transposon in the chromosome of *S. pyogenes* transconjugants. In addition, because DP1333 has a copy of Tn*5252* but not Rx1 (TableI), the frequency of Em^r transformants of DP1333 should be greater (due to homologous recombination) than that of Rx1. The Em^r transformants of DP1333 were three-to-four orders of magnitude greater then those of Rx1 confirming that

pVA891 had in fact inserted within the transposon (Table XI). No Em^r or Cm^r transformants of Rx1 or DP1333 were obtained with chromosomal DNA from *S. pyogenes.*

Confirmation of Conjugal Transfer of SP1254 Insertion Mutant to *S. pyogenes* by Southern Hybridization

The transfer-proficient insertion-mutant strain, SP1254, is able to transfer to *S. pyogenes* in filter mating as mentioned before (Table X). To physically confirm the presence of Tn*5252* in the *S. pyogenes* Em^r transconjugants, chromosomal DNA from one of the Em^r Cm^r transconjugants was digested with *Eco*RI and *Hin*dIII and probed with ^{32}P-labeled pDR5 DNA. As indicated in Figure 35, chromosomal DNA from *S. pyogenes* did not react with the probe (lane a) whereas SP1000 (lane b), SP1254 (lane c), and the putative transconjugant (lane d) hybridized with the probe as expected. A single 4.5-kb *Eco*RI fragment of SP1000 (pattern A, lane b) gave rise to two, 6.0 kb and 4.5 kb, fragments in SP1254 (pattern A, lane c). The *Hin*dIII fragments (pattern B) of SP1000 and SP1254 were as described previously (see Figure 21). As evident from the autoradiogram, the *S. pyogenes* transconjugant of SP1254, designated SY152, did in fact react with the probe and give fragments identical to those of SP1254. This confirmed the presence of Tn*5252* on the chromosome of *S. pyogenes.*

TABLE XI

CONFIRMATION OF INSERTION OF pVA891 WITHIN Tn*5252*

	Em^r transformants /recipient	
Donor DNA	Rx1	DP1333
SY152	3.9×10^{-7}	1.7×10^{-4}
SY153	1.6×10^{-8}	1.5×10^{-4}
SY154	1.8×10^{-8}	5.4×10^{-4}
SY158	1.8×10^{-8}	9.2×10^{-5}
SY159	2.4×10^{-7}	1.7×10^{-4}
SP1000	5.4×10^{-7}	6.0×10^{-4}

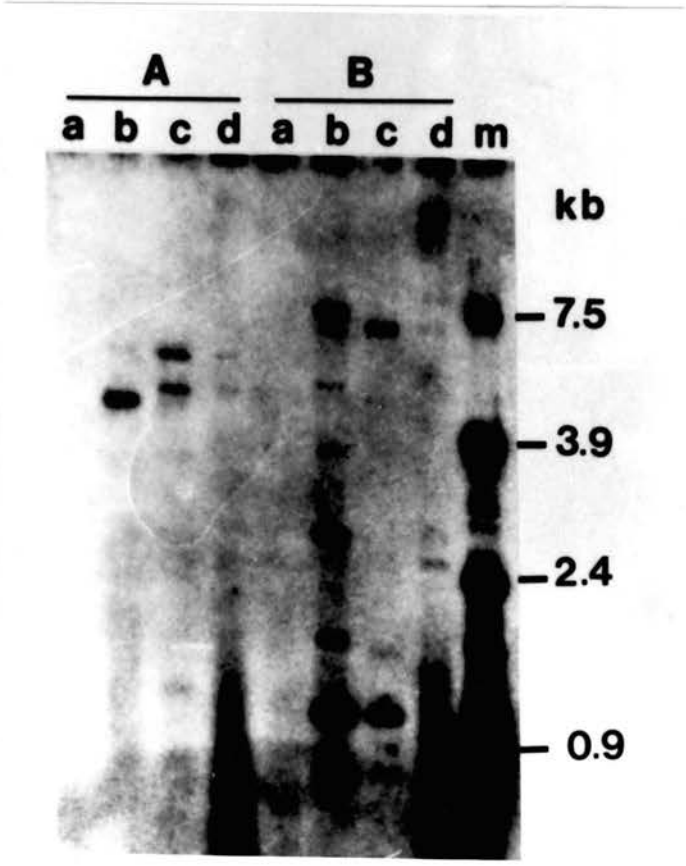


Figure 35. Analyses of *S. pyogenes* Transconjugants of Insertion Mutant Strain SP1254 by Southern Hybridization. *Eco*RI (A) and *Hind*III (B) digested chromosomal DNAs were probed with ^{32}P-labeled pDR5. a, *S. pyogenes*; b, SP1000; c, SP1254; d, Em^r clone of *S. pyogenes*. m, molecular weight marker. The clone in lane d was designated as SY152.

Confirmation of Conjugal Transfer of SP1255 Insertion Mutant to *S. pyogenes* by Southern Hybridization

The transfer proficient insertion strain, SP1255, was mobilized into *S. pyogenes* recipients via conjugation. Two putative transconjugants were examined for the presence of Tn*5252* in blot hybridization experiments. Chromosomal DNAs from wild-type *S. pyogenes* strain, SP1000, SP1255, and the two transconjugants were digested with *Eco*RI and *Hin*dIII, transferred to a nylon membrane and probed with radioactively-labeled pDR2 DNA. As evident from Figure 36, *S. pyogenes* did not react with the probe (lane a) whereas the two transconjugants (lanes d and e) gave identical hybridization patterns as SP1255 indicating the transfer of Tn*5252* to those *S. pyogenes* recipients.

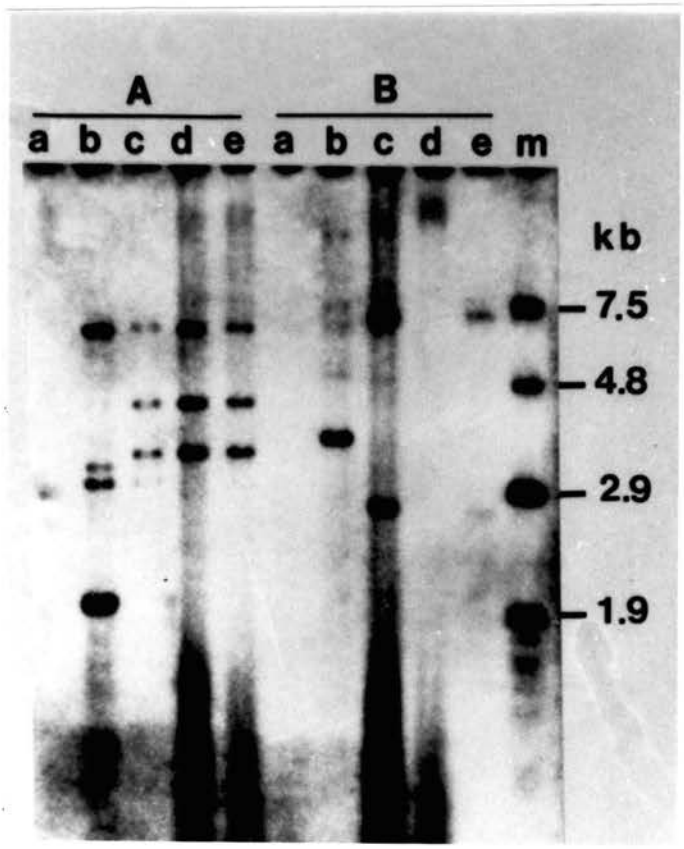


Figure 36. Analyses of *S. pyogenes* Transconjugants of Insertion Mutant Strain SP1255 by Southern Hybridization. *Eco*RI (A) and *Hin*dIII (B) digested chromosomal DNAs were probed with ^{32}P-labeled pDR5. a, *S. pyogenes*; b, SP1000; c, SP1254; d and e, Em^r clones of *S. pyogenes*. m, molecular weight marker. The clone in lane d was designated as SY153.

Summary of Deletion and Insertion Mutagenesis of Tn*5252*

The composite nature of Tn*5253* and its derivatives are shown in Figure 37. Separation of Tn*5251* from Tn*5253* results in Tn*5252* (3). The transfer functions of Tn*5252* are not impaired when the *cat* region spontaneously cures (70).

All of the six deletions (indicated with shaded boxes, A through F) and nine insertion mutations (indicated with numbers) within Tn*5252* are shown in Figure 38. The conjugal transfer properties of each mutant derivative of Tn*5252* are given as Tra^+ (transfer proficient) and Tra^- (transfer deficient) within the parentheses. Table VIII shows the coordinates of restriction endonuclease sites present in Tn*5252* from SP1000. The *Bam*HI site immediately outside of the left junction of the transposon on the recipient chromosome is arbitarily chosen as coordinate 0.0.

The recombinant plasmids used to create deletions and insertions within Tn*5252* are listed in Table IX. Transposon DNA coordinates carried on the vector plasmids, restriction enzyme sites, coordinates of deletions and insertions are also indicated in Table IX.

A schematic representation of the recombinant plasmids used in experiments with deletion and insertion mutations, sequencing, and blot hybridization is given in Figure 9.

Figure 37. Composite Nature of Tn*5253* and Its Derivatives. a and b indicate left and right chromosomal regions flanking the transposon. Separation of Tn*5251* from Tn*5253* results in Tn*5252*. The *cat* region located at the left site within the element is flanked by direct repeats of 1.7 kb and often spontaneously cures. The transfer function of the remaining Tn5252Δ*cat* is not impaired.

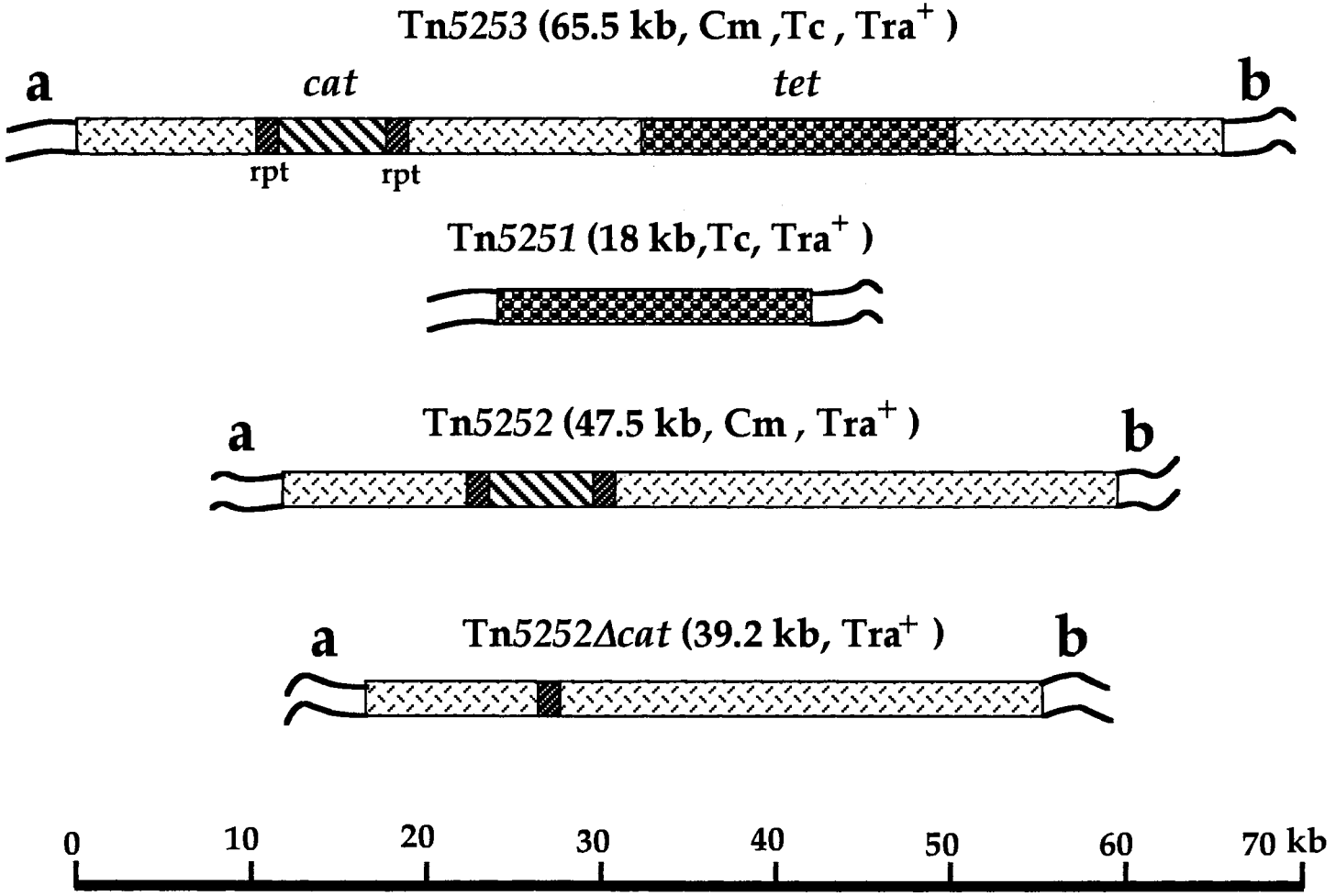
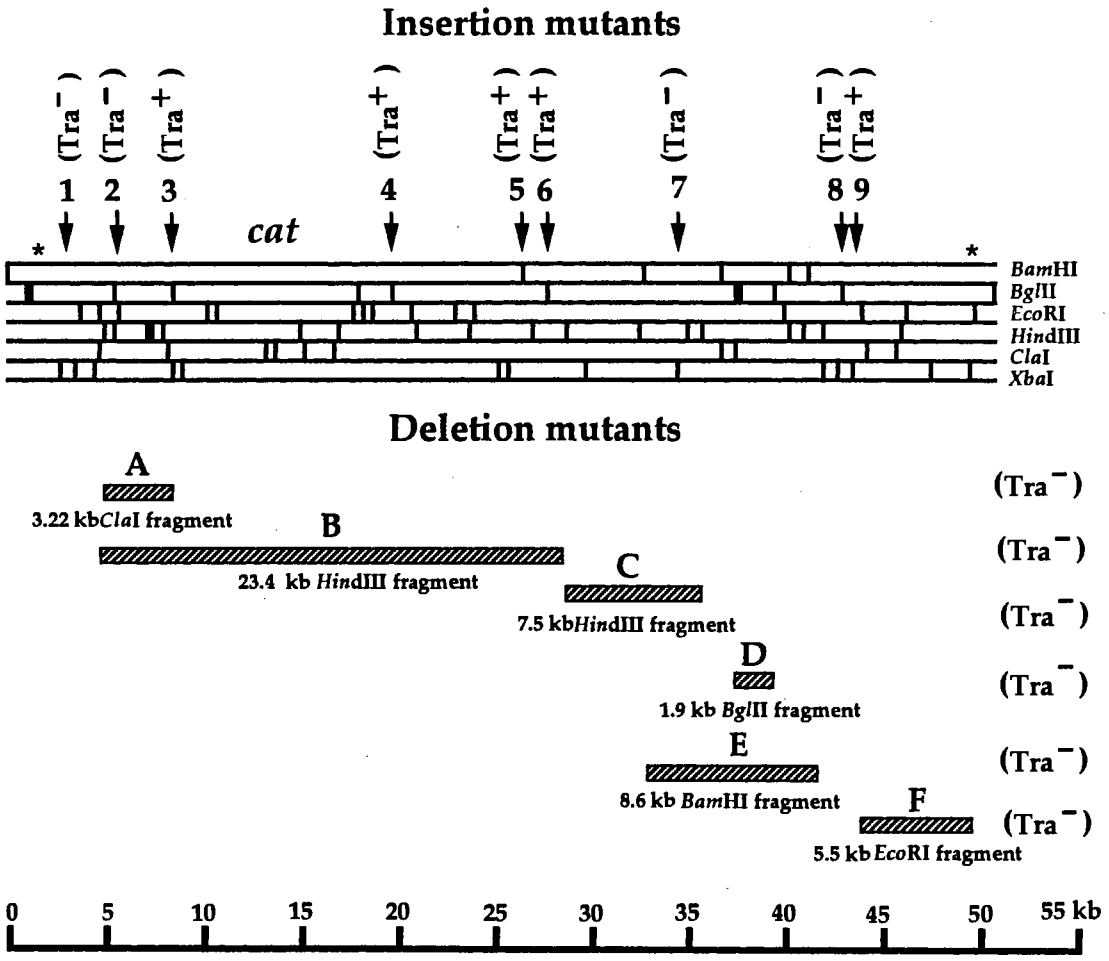

Tn5253 (65.5 kb, Cm ,Tc , Tra+)
a
cat
tet
b
rpt
rpt
Tn5251 (18 kb,Tc, Tra+)
a
Tn5252 (47.5 kb, Cm , Tra+)
b
a
Tn5252Δcat (39.2 kb, Tra+)
b
0
10
20
30
40
50
60
70 kb

Figure 38. Transfer-related Regions in Tn*5252*. The asterisks mark the end of the transposon. The numbers refer to the insertion mutant strains and the arrows point to the insertion of the heterologous drug resistance gene. The numbers representing insertion strains as follows: 1, SP1253; 2, SP1252; 3, SP1254; 4, SP1255; 5, SP1257; 6, SP1258; 7, SP1256; 8, SP1259; and 9, SP1260. The boxes show the segments of DNA deleted within Tn*5252*. The deletion strains are indicated as A through F. A, SP1200; B, SP1201; C, SP1203; D, SP1204; and F, SP1205. The transfer properties of the mutant strains are indicated as Tra^+ (transfer proficient) and Tra^- (transfer deficient).


Insertion mutants
(Tra−)
(Tra−)
(Tra+)
(Tra+)
(Tra+)
(Tra−)
(Tra−)
(Tra+)
1 2 3 4 5 6 7 8 9
cat
BamHI
BglII
EcoRI
HindIII
ClaI
XbaI
Deletion mutants
A
3.22 kb ClaI fragment
B
23.4 kb HindIII fragment
C
7.5 kb HindIII fragment
D
1.9 kb BglII fragment
E
8.6 kb BamHI fragment
F
5.5 kb EcoRI fragment
(Tra−)
(Tra−)
(Tra−)
(Tra−)
(Tra−)
(Tra−)
0 5 10 15 20 25 30 35 40 45 50 55 kb

DNA Sequence Analysis of a Transfer-Related Region of Tn*5252*

The major goal of this study was to establish the functional map of Tn*5252* by localizing its transfer-related regions and help future efforts to identify these regions, and/or genes at the molecular level. One of the projects was to sequence those regions thought to be involved in the transfer of the transposon. Srivinas and I sequenced a 3.2-kb region flanked by *Xba*I sites (coordinates 4.6 to 8.0) where a drug-resistance gene had been previously inserted (1). This insertion was found to impair the conjugal transfer of Tn*5252*. The 3.2 kb *Xba*I fragment on the left end of Tn*5252* was cloned into pBluescript SK+ vector to create the recombinant plasmid pSP108. The plasmid pSP108 was treated with exonuclease III and S1 nuclease to construct a nested set of deletion derivatives. A schematic representation of these constructs and the sequencing strategy is given in Figure 39. Double-stranded and single stranded templates were prepared from the resulting deletion derivative plasmids and used in sequencing as described in the material and methods section. Both strands of the DNA fragment were sequenced.

The sequence was found to be 3272 base pairs long. Analysis of the nucleotide sequence revealed three open-reading frames (ORFs) designated ORF 4, ORF 7, ORF 8. Of the three, ORF 4 was further analyzed and compared for similarity with other DNA sequences in different data banks. The ORF 4 is 849 base pairs. It is predicted to encode a protein of 282 amino acids. A single *Bgl*II site at which the drug-resistance gene had been inserted is located in the central region of this gene (Figure 40). The deduced amino acid sequence of ORF 4 was also analyzed for similarities with other protein sequences in data bases. Data bank analyses revealed

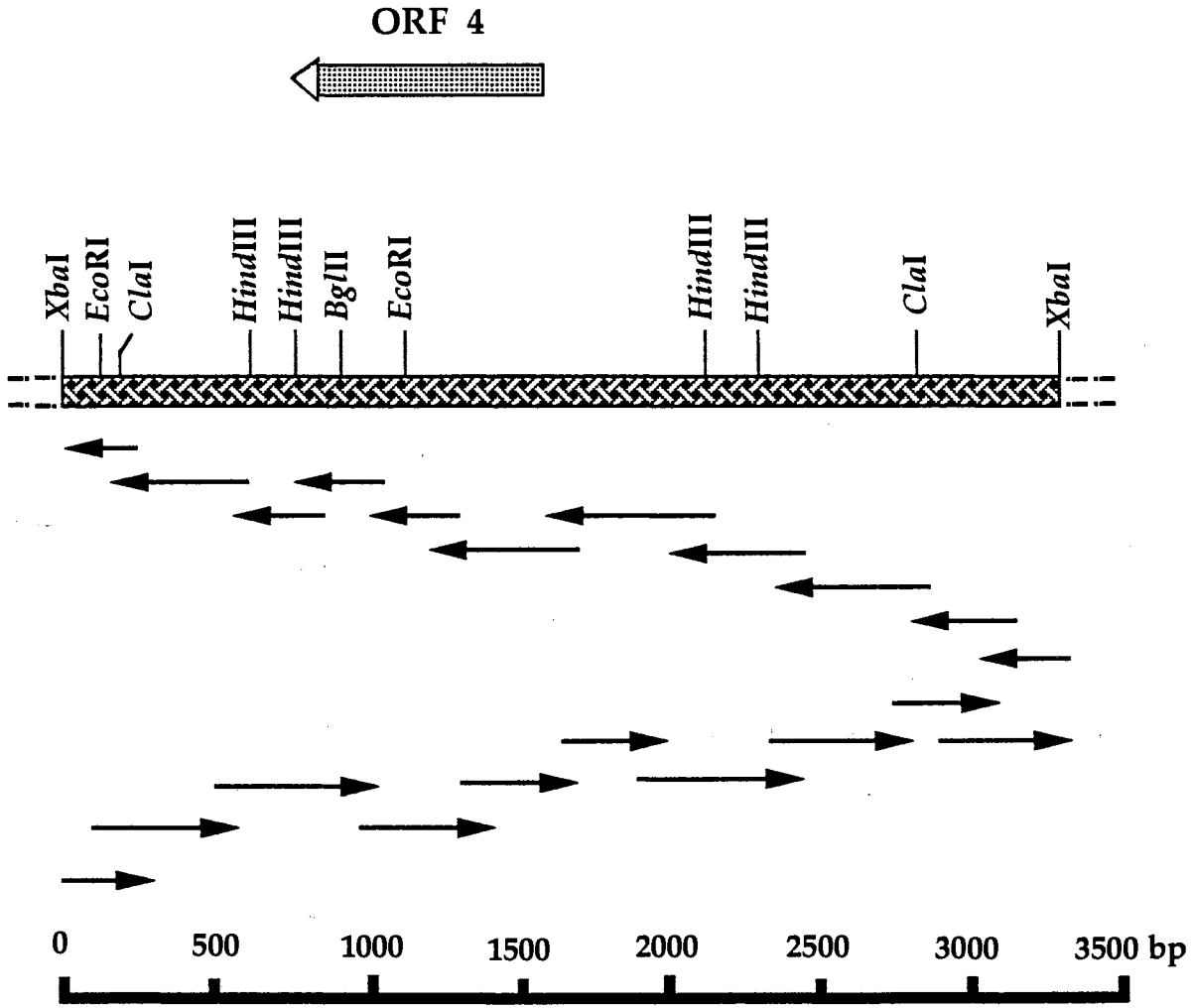


Figure 39. Sequencing Strategy of 3.2 kb *Xba*I Fragment.

Figure 40. Nucleotide Sequence of 3.2 kb *Xba*I Fragment (coordinates: 4.8-8.0) of Tn*5252*. The codons and deduced amino acid sequence of ORF 4 are indicated. The BglII site where pVA891 was inserted within the ORF 4 is also indicated.

*Xba*I

```
   1  AAG CTT CTA GAA CAT TGC TAG AAG AAA CTC ATT GGT GGT CAT TCA GCT GGA GGA
  55  TTA GAT GGA CTA AAA TAA TGA ATC GTT CTT ATT TAC CTT TTG AGC CTG CCC GAG
 109  TAT ACT AGG ATC GTG GCA TGG CTA AGT GGA TGG GCT TCT TTT ATC AGA ACA TTC
 163  CAG TTC TCT TTG GGC AGA AAA AAA TAA AGA AGA TAT CTC CAT TTC CCT ATC ATT
 217  GGA AGA GAA AGA GAT TTC TAT TTG TTC GTC AAC TTT ATA CGA ATG TAT TCC CTG
 271  CAA CTT TTG TTT TTA AGT TTT CTA ATC AAA GAA AAG TAG TAT CAG GTA TTG TTA
 325  AAG AGA TTG GAA AAG AGT TTA TAT CTA TAA AAT CAG ACA CTG GTT TTC TTC GAT
 379  TAA AAT GGG AAG ATA TAC TCG ATA TAC AGA TAG AAG GGG AGG AAT TAC ATG AAT
 433  CGT AAA GAA TTA TAT CGA TCT GAT CAA ATT ACC GCT AGA TTA TTT TTC AGA TTC
 487  TTA TTT ACA GTT TGA GTC AGA TTT TTA CAA GTA TTC AGC TTT AGA TAT ACC ATT
 541  AAC ATT TAT CAC AGA TGA TAT TTT ACG CAC AAT GGC TAT GTC TCA AAA ACA TTA
 595  TTT TAA ACT TAA CAA AAG TAA ATC TTT AGA CGG TCG TGA TCA TTA CTT TGT TTT
 649  TTC TAT CAA GAT GAA CAA AGA CAG TAG TGG TAT TAG ACA GTA TGA ATA TCA GAG
 703  ACA TTG TTT TAA TTT GTA AGA GTC CGA CAG GCT CTT TTT TCT CGT CGA TAA TTT
 757  TAT CAA AAA GTA TTT GTT ATA CTT TTT TTA ATT TAG ATT TAT CTT GGG GTT TGG
 811  GGG GCG TGC CAC CAC ATT TTC ATA TCG TTT GTT TTT TAG AAC CGT GAG GTT TGA
 865  AAT GGC GGC GAT ATG ATT TTT GGG ATA TTG TGG ACA CAA TAT CTG AGC TCG CAA
 919  AGC CAT ACA AAA ATG TTG AAT CTA TTT TGT AAA ACG TAC TGA CAG TGT ATG TAA
 973  GCT TAC ATT GCC AGT ACA AGT ATC TAT GAG AGA GGA TAA ATC ATA TGA AAA GAA
1027  ATA TAC GTA GTA TTC GGA AAC AAT TTC GCT TAA CAG AAA CGG AAG AAA AAC AAA
1081  TAC TAG ATT TAA TGA GAG AGA AAG GAG ATG ATA ATT TTT CTG ATT TTC TCC GTA
1135  AAA GCT TAC TAT TAT CTG ATG GAC AAA AAC AGA TGG AAA AAT GGT TCA ACC TTT
1189  GGA AAA AAA ACA AAA GTT GGA ACA AAT TAG TCG TGA TGT TCA TGA AGT ATT CAT
1243  AAT TGC TAA AAC AAA TCA TCA GGT TAC TCA CGA ACA TGT TTC TAT TTT GTT AAC
1297  TTG TAT TCA GGA ATT AAT TAA AGA GGT AGA AAA AAC AGG TCC TCT TAG TGA AGA
1351  TTT TAG TAA TAA ATA CAT GAG GTA GTA GAG TGG AGC ACA GAT ATA GAA CAA ATT
1405  TAA AAA AAG CCT TTC TAT CTG ATC TAG AAT TAG TCA AAT TGA ATG AAA ATA TCT
1459  GAA AAA GCA ACT GCT TAT CAT TCT CAG AAT ATG CTA GAC GAA CTC TAC TAG ATC
1513  CTG GTA TGA ATT TTA TCA CCA TTG ATA CAA ATA GTT ATC AAG ATT TGA TTT TTG
1567  AAT TAA AAC GAA TCG GAA ATA ATA TTA ATC AAA TAG CCA GAA GCA TAA ATT ATT
1621  CGA ATT TAA TAA CGG AAG TTG AAT TAA ATG AGT TGA GAA AAG GTA TAG AAG AGT
1675  TAA TAG TAG AAG TGG AGA AAG ATT TTC TTA TTC GAT CTG AAA AAT TGA GGA AAT
   1        M   V   I   T   K   H   F   A   I   H   G   K   N   Y   R   S   K
1729  TTT ATG GTC ATC ACT AAA CAC TTT GCG ATT CAT GGA AAA AAT TAT CGT AGT AAA
  18    L   I   K   Y   I   L   N   P   S   K   T   K   D   L   T   L   V   S
1783  CTA ATC AAA TAT ATT TTG AAT CCA AGT AAA ACA AAA GAT CTA ACA CTA GTT TCA
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  36   D   F   G   M   R   N   Y   L   D   F   P   S   Y   K   E   L   V   K
1837 GAT TTT GGT ATG AGA AAT TAT TTA GAT TTT CCT AGT TAT AAA GAA CTA GTG AAG

  54   M   Y   N   D   N   F   L   S   N   D   T   L   Y   E   F   R   H   D
1891 ATG TAC AAT GAT AAT TTT TTA AGT AAT GAT ACT CTT TAT GAA TTT CGT CAT GAT

  72   R   Q   E   V   N   Q   R   K   I   H   S   H   H   I   I   Q   S   F
1945 AGG CAA GAA GTA AAT CAA CGA AAA ATT CAT TCT CAT CAC ATC ATT CAG TCC TTT

  90   S   P   D   D   H   L   T   P   E   Q   I   N   R   I   G   Y   E   A
1999 TCT CCA GAT GAC CAT CTC ACT CCT GAA CAA ATC AAT CGA ATT GGT TAT GAG GCA

 108   A   K   E   L   T   V   G   R   F   R   F   I   V   A   T   H   V   D
2053 GCT AAA GAG TTG ACA GTA GGT AGA TTT CGT TTT ATT GTA GCA ACT CAT GTC GAT

 126   K   G   H   I   H   N   H   I   I   L   N   S   I   D   Q   N   S   D
2107 AAA GGT CAT ATC CAC AAT CAC ATC ATC CTA AAT TCA ATT GAT CAG AAT TCT GAT

 144   K   K   F   L   W   D   Y   K   A   E   H   N   L   R   M   V   S   D
2161 AAA AAG TTT CTA TGG GAT TAT AAG GCA GAA CAT AAT CTA CGA ATG GTT TCT GAT

 162   R   L   S   K   I   A   G   A   K   I   I   E   N   R   Y   S   H   R
2215 CGT CTT TCA AAA ATT GCA GGG GCA AAA ATT ATA GAA AAT CGT TAT TCG CAT CGT

 180   Q   Y   E   V   Y   R   K   T   N   Y   K   Y   E   I   K   Q   R   V
2269 CAG TAT GAA GTT TAT CGC AAA ACA AAT TAC AAA TAT GAA ATA AAA CAA CGG GTA
                                                 BglII
 198   Y   F   L   I   E   N   S   K   N   F   E   D   L   K   K   K   A   K
2323 TAT TTT CTA ATC GAG AAC TCG AAA AAT TTT GAA GAT CTT AAG AAA AAA GCT AAA

 216   A   L   H   L   K   I   D   F   R   H   K   H   V   T   Y   F   M   T
2377 GCT TTA CAT TTA AAA ATT GAT TTT AGA CAC AAG CAT GTT ACT TAT TTT ATG ACT

 234   D   S   N   M   K   Q   V   V   R   D   S   K   L   S   R   K   Q   P
2431 GAT TCA AAT ATG AAA CAA GTC GTA CGT GAT AGT AAA TTG AGT AGA AAA CAA CCT

 252   Y   N   E   T   Y   F   E   K   K   L   C   S   K   G   N   H   K   H
2485 TAT AAT GAA ACT TAT TTT GAG AAA AAG CTT TGT TCA AAG GGA AAT CAT AAA CAT

 270   I   R   I   F   T   S   E   N   E   E   Y   E   *
2539 ATT AGA ATT TTT ACT TCC GAA AAT GAA GAA TAT GAA TGA ATT GAT TCA ACG AGC

2647 TGA TCG GGA TTA AGC TTG CAG AGC AGG AAT TGG TAA AAA CGC AAT CTG TAT AGT

2701 GTT AGT TAT TTT CAA GAC TAT TTT AAT AAC AAA AAT GAA ACT TTT GTC TTA GAT

2755 AAT AAA AAT TTA GTT GAA CTT TAC AAT GAA GAA AAG ATA ATT AAA GAA AAA GAG

2809 TTG CCG TCA GAA GAG ATG GTA TGG AAA TCT TAT CAA GAT TTC AAG AGA AAT AGA

2863 GAT GCT GTT CAT GAG TTT GAA GTA GAG TTG AAT CTT AAT CAA ATA GAA GAA GTA

2917 GTA GAG CAT GGA ATT TAC ATT AAG GTA CAG TTT GGT ATC GAC AAG AAG GAC TTA

2971 TTT TTG TAC CAA ATA TTC AGA TCA ATA TGG AAG AAG AAA AAG TTA AAG TAT TTC

3025 TCA GAG AAA CTA GTT CTT ACT ATG TAT ATC ATA AAG ATT CAG TAG TAA AAA AAT

3079 CGA TTT ATG AAA GGT AAA ACT TTG ATT AGA CAA TTT AAT CTT CAG TAT GAA CCA

3133 CAG TAT ATG TAT AGA AGA ATT CCT CTT AGC AAA ATT AAA GAA AAA ATA GAA CAA

3187 TTA GAT TTT CTT ATA TCT GCG GAA AAT AGT TCG AAT GAT TTT GAA GAT ATA ACA
                                    XbaI
3241 AAT GAT TTC ATT GCC CAA ATA TCA TAT CTA GA
```

significant similarity between the deduced amino acid sequence of ORF 4 to relaxation proteins of *S. aureus* plasmids. The highest similarity (indicated by smallest Poisson probability) obtained were $2.8x10^{-10}$ (40% identity over 62 amino acids), $3.9x10^{-9}$ (30% identity over 80 amino acids) and $1.5x10^{-9}$ (31% identity over 58 amino acids) for the plasmids pC223, pC221 and pS194 respectively.

The extreme carboxyl terminal and amino terminal regions of these relaxation proteins seem to be considerably divergent. Hence, only the conserved regions of the relaxation protein of pS194, pC221, and pC223 and the predicted amino acid sequence of a part of ORF 4 were compared (Figure 41).

Relaxation protein is involved in the formation of relaxosome, a nucleoprotein complex, which is believed to be involved in the strand specific cleavage activity of protein complexes formed during the conjugal transfer of gram-negative plasmids such as IncP, colicin and RP4 (80). The functions of relaxosomes in the staphylococcal plasmids have not been documented. However, the staphylococcal plasmids seem to replicate via a rolling circle mechanism similar to those of gram-negative plasmids. This suggests that they may be involved in similar activities during the plasmid replication. Whether this model can be applied to conjugative transposons, such as Tn*5252*, is yet to be determined. Prevention of the conjugal transposition of Tn*5252* following insertion mutagenesis within this gene suggests that it may play a role in this system as well. More studies are needed to understand the function of this protein in the process of transposition.

```
                  *      +  * ** ** * *****+*****  + ***
ORF-4   107 -AAKELTGGRFRFIVATHVDKGHIHNHIILNSIDQNSDKKFLWDY-
RLX1     78 -ELAKKIAPDYQVAVYTHTDKDHYHNHIIINSVNLETGNKYQSNK-
RLX2     78 -ELAEKIAPNHQVAVYTHTDKDHYHNHIVINSVDLETGKKYQSNK-
RLX3     78 -ELAEKIAPNHQVAVYTHNDTDHVHNHIVINSIDLETGKKFNNNK-
```

Figure 41. Comparative Analysis of the Conserved Regions of Relaxation Proteins of *S. aureus* Plasmids to the Predicted Amino Acid Sequence of a Part of ORF 4. RLX 1 is from the Plasmid, pS194; RLX 2 from pC221, and RLX 3 from pC223.

In the evolution and rearrangement of prokaryotic and eukaryotic genomes, transposable elements are probably one of the most powerful driving forces. Transposable elements mediate genetic exchange not only between replicons within a cell but also between members of related, distantly related, or even unrelated genera. The significance of gene transfer and rearrangements of the genomes is that it provides a mechanism for increasing the diversity of the gene pool available for bacterial evolution.

Composite Nature of Tn*5253*

This study was initiated to gain understanding of the structural and genetic organization of the ΩBM6001 element (now termed Tn*5253*) mediating multiple antibiotic resistance. Antibiotic resistance in bacteria is mostly associated with plasmids. As in most bacteria, resistance plasmids are common in streptococci with the exception of pneumococci. However, pneumococci can receive and stably maintain a variety of plasmids from other streptococci (70). With the worldwide emergence of multiple-antibiotic resistance in the late 1970's (39), a new class of mobile chromosomal elements conveniently termed "conjugative transposons" have been identified, they have been shown to be the major carriers of the multiple-antibiotic resistance (8, 23, 25, 37, 41, 72). Besides transfer functions, many of the streptococcal transposons may also carry one or more antibiotic-resistance determinants, and almost all carry a homologous tetracycline-resistance determinant of the type *tetM* (7). The other resistance determinants include chloramphenicol (*cat*), erythromycin (*erm*), and kanamycin (*aph*A) carried on the conjugative transposons of different

species of streptococci. Based on their size, these conjugative transposons could be grouped into two types; one ranging from 16-to-25 kb such as Tn*916* (*tet*) from *E. faecalis* DS16 (25) and Tn*1545* (*tet erm aphA*) from *S. pneumoniae* BM4200 (21), and the others around 60 kb such as Tn*3701* (*tet erm*) from *S. pyogenes* A454 (42), Tn*3951* from *S. agalactiae* B109 (38, 72), and Tn*5253* (*cat tet*) from *S.pneumoniae* BM6001 (70, 76, 77).

The results presented in this study demonstrate that the *tet* determinant of Tn*5253* is homologous to Tn*916* and related *tet* elements and is an independent conjugative transposon. This conjugative transposon, designated Tn*5251*, exhibited intercellular conjugal-transfer properties as well as intracellular transposition when it was removed from the larger transposon, Tn*5253*. The data obtained in this study were supported with subsequent findings of Le Bouguenec and coworkers (43). They have also identified a transposable element, *Tn3703*, within the larger (>50 kb) conjugative transposon, Tn3701, carried on the chromosome of *S. pyogenes* A454 (41, 42). Tn*3701*, Tn*5251*, Tn*916* and Tn*1545* were shown to not only share extensive homology in DNA-DNA hybridization studies but to also have structural similarities as determined by restriction map analyses (42, 43).

In addition to the identification of Tn*5251*, *Tn5253* contains Tn*5252*, which is made up of the sequences beyond Tn*5251* within Tn*5253* (3). This element is also capable of transfer via conjugation within pneumococci and other streptococci. These results clearly indicated that Tn*5251* does not play a mechanistic role in the conjugal transfer of the larger transposon, Tn*5252*. Horoud *et al.* (35) examined the insertion of Tn*3701* in different streptococcal recipients of groups A, B, C, G, and D, and *S. pneumoniae*. In all thirteen streptococcal transconjugants belonging to six different species

and in one of the twelve *E. faecalis* transconjugants, Tn*3701* did not show any apparent structural changes. Each of the seven *E. faecalis* transconjugants carried one copy of Tn*3701* and an additional copy of Tn*3703*. Four *E. faecalis* transconjugants were found to carry Tn*3703* alone and inserted at different sites in the chromosome. Moreover, Tn*3703* was capable of intracellular transposition onto the hemolysin plasmid pIP964 (34) suggested that Tn*3701* is a composite transposon and the conjugal transfer related genes of Tn*3701* are located outside of Tn*3703* (35). However, unlike Tn*5251*, Tn*3703* was not able to further transfer via conjugation from *E. faecalis* donors to other species suggesting that the host specificity of Tn*3703* was more restricted than that of Tn*3701*.

The data presented in this study also suggested that Tn*5251* has some degree of host specificity. The conjugal transposition of Tn*5251* alone from a *S. pneumoniae* donor to an *E. faecalis* recipient was unsuccessful. On the other hand, Tn*5251* could be introduced into *E. faecalis* as a part of the parental transposon, Tn*5253*. When these *E. faecalis* transconjugants were used as donors in filter matings with pneumococcal recipients, Tn*5251* seemed to excise from Tn*5253* and insert at different sites in the recipient chromosome. The independent transposition of Tn*5251* from Tn*5253* onto the chromosome of pneumococci had not been observed previously (70, 71). These findings provided additional genetic evidence that Tn*5253* is a composite transposon made up of two independent transposons.

As evident from these data, the behavioral differences in the independent transposition and target preference of the composite transposons may be under the influence of some as yet unknown host factor. In agreement with Le Bouguenec and coworkers (43), these results seem to suggest that smaller elements such as Tn*5251* were added at a later time to

Tn*5252*-class elements to establish composite structures such as Tn*5253*. Therefore, instead of Tn*916*-like elements, Tn*5252*-like elements should be considered progenitors of the larger transposons. In addition, the orientation of Tn*916*-like elements in the composite transposons, Tn*5253*, Tn*3701* and Tn*3951*, were found to be variable, indicating that these elements might not have been the progenitors of the composite transposons but inserted later within preexisting genetic elements.

Based on blot hybridization and transformation properties, the *cat* determinant of Tn*5252* is flanked by directly repeated sequences of about 1.7 kb which seem to be present in the chromosome of the wild-type pneumococcal strain Rx1 (70, 77). Also, another region contained within a 2.4-kb *Eco*RI fragment in Tn*5252* between coordinates 44.0 and 46.4 was found to be present in the wild-type chromosome in two places. These sequences are thought to be IS-like elements (Vijayakumar, personal communication). The spontaneous deletion of *cat* can be accounted for as a result of recombination between the two direct repeats (70). Using chromosomal DNAs from streptococcal strains carrying Tn*5253*, Tn*3701* and Tn*3951*, Smith (70) was able to transform Rx1 cells for *cat*, independent from the other drug resistance determinants (i.e., *erm* and *tet*) in the transposon strongly suggesting that the *cat* determinant from other elements had properties very similar to that *cat* of Tn*5253*. Based on DNA-DNA hybridization studies, Le Bouguenec *et al.* (43) recently showed that Tn*3701*, Tn*3951* and Tn*5253* share extensive homology not only around the internal Tn*916*-like segments but also to the regions outside of these sequences including the *cat* determinant. The presence of Tn*5252*-like sequences outside of the *cat* and *tet* determinants on the chromosome of many antibiotic-resistant isolates of various streptococcal species (43)

supported the idea that these drug-resistance chromosomes might have autoaccumulated under the selective pressure of antibiotics, established a stable relationship, and given rise to the conjugative transposons of the streptococci.

Transfer-related Regions of Tn*5252*

The major focus of this work was to identify the regions involved in the self-transmission of Tn*5252* which has been chiefly responsible for the dissemination of antibiotic resistance among the medically important pathogenic streptococci. The multidrug resistance in clinical streptococci has been identified to be associated with one of the two types of transposons, Tn*916* and Tn*5252*. Of these two, Tn*916* and the related transposons, Tn*1545*, have been studied and characterized in some detail. On the other hand, Tn*5252*, believed to be distinctly different from Tn*916*-like transposons, is only now being analyzed. Cloning of the entire set of DNA fragments associated with Tn*5252* (76) made it possible for me to carry out the outlined genetic studies. To understand the conjugal transposition properties of Tn*5252*, the cloned DNA fragments were used in *in vitro* manipulations to mutate specific regions within the transposon in a search for genes possibly encoding products involved in conjugal transposition.

As an initial step, defined DNA segments were removed from the insert portion of recombinant plasmids and replaced with an heterologous DNA carrying a selectable drug-resistance marker, such as pVA891. The mutated fragment was introduced into a pneumococcal recipient carrying Tn*5252* by transformation for *in vivo* mutagenesis of the transposon. This

type of strategy differs from that of insertion-duplication mutagenesis as used previously to generate recombinant plasmids carrying the entire Tn*5253* DNA segments in *E. coli* (76).

A similar strategy was followed to insert the drug-resistance gene at various restriction sites *in vitro*. The conjugal transfer ability of each mutant derivative of Tn*5252* was tested in filter mating experiments with *S. pyogenes* recipients. Of the nine insertion mutants five were able to transfer to *S. pyogenes* recipients at a frequency comparable to that of non-mutated Tn*5252*. On the other hand, all six deletion mutants were found to be transfer deficient. The six deletions covered about 90% of Tn*5252* including the entire right junction except the extreme left end region (coordinates 0.0 to 4.8) and a 2.4-kb region (coordinates 41.6 to 44.0) at the right end of the transposon. Insertions within these regions (coordinates 3.1 and 42.7 respectively) were introduced.

The transfer proficient insertion mutants of Tn*5252* were confirmed by either transformation experiments or in subsequent filter matings for their donor ability (data not shown). Two of them were also analyzed in blot hybridization. Since there is no report of the natural transferability of *S. pyogenes* strains or extensive homology between *S. pyogenes* and pneumococci, all five transconjugants of *tra*-proficient Tn*5252* would be expected to be due to conjugation.

The experimental data seem to indicate that the *tra* genes are clustered at the ends, particularly at the left end of the transposon. It is worth noting that insertions in the central regions of the element did not affect its conjugal transposition functions strongly. These data suggest that this segment of DNA can acquire insertions such as Tn*5251* or the *cat* determinant without affecting transfer.

These data help to target specific genes that are involved in specific functions in Tn*5252*. As suggested, a large portion of the mobile elements, such as conjugative plasmids and conjugative transposons, is devoted to the conjugal Tra functions. The *tra* genes of pIP501, a streptococcal broad-host-range conjugative plasmid, encompass more than half of its size, 30.2 kb (40). In Tn*916* conjugal transfer genes seemed to account for more than 50% of the DNA, whereas the genes required for intracellular transposition (excision/integration) are encoded by an additional 10-15% of its DNA (63, 82). The genetic data indicated that some regions, around the *tet* determinant of Tn*916*, seemed to be nonessential for the transfer of Tn*916*. The functional features of these regions has not been identified yet. In agreement with these data, some regions within Tn*5252* might be involved in some other functions. The loss of the *cat* region does not impair the conjugal transfer functions of the remaining sequences in Tn*5252* (70).

There has been little information on the evolution of conjugative systems. While there is no direct evidence, the transfer-related genes of conjugative transposons are thought to be related to those of the conjugative plasmids (18). The DNA sequence and predicted protein sequence analysis revealed some similarities with the Int and Xis families protein of lambda phage and Tn*916* and Tn*1545* (20). Similarities between these phage-related proteins and Tra proteins of the conjugative plasmid pSAM2 of *Streptomyces ambofacience* has been reported (53, 54). These data suggest that the origin of the *tra* genes of conjugative transposons and those of conjugative plasmids, and perhaps of lambda phages may have originated from a common ancestor.

The *tetM* type of resistance common to all conjugative transposons did not show homology to *tet* genes on plasmids from either gram-positive

or gram-negative bacteria (70). It has been shown that the *aphA* gene found in Tn*1545* is homologous to that on the *S. aureus* plasmid pSH2 (70). Furthermore the *cat* gene of Tn*5252* has been shown to be homologous to the staphylococcal plasmid pC194 (70). These results support the idea of autoaccumulation of various genetic units into a prototype element such as Tn*5252* to form larger conjugative transposons. Analysis of the DNA-sequence data inform in this study showed some similarity to the relaxation proteins of staphylococcal plasmids pS194 and pC223, indicating the possibility of autoaccumulation of various genes within conjugative transposons like Tn*5252*. However, these data are insufficient to draw a conclusion on the origin and evolution of drug-resistance determinants of conjugative transposons.

Besides the transfer-related genes and drug resistance determinants of streptococcal conjugative transposons, it is likely that some other genes are associated with the transposon without interfering with its integrity.

In *S. aureus*, a wide variety of virulence genes such as enterotoxin B gene, a cause of staphylococcal food poisoning has been found to be carried by the transposable elements (64). The presence of this type of gene within streptococcal conjugative transposons has not been reported. However, it may be possible that some of these genes carried on transposable elements had inserted within the larger transposons such as Tn*5252* without interrupting its transfer functions. The presence of five different unrelated Tra regions within Tn*5252* indicates this type of evolution of the transposons may have taken place.

At present, very little is known about the mechanism of conjugal gene transfer in gram-positive bacteria. In the case of conjugative transposons, only two, Tn*916* and Tn*1545*, have been studied at the genetic level. Recent

studies indicate that transposition properties of these transposons are very similar (20). The transposition event is initiated by an excision event created by staggered nicks at the termini of the transposon followed by formation of a covalently-closed supercoil molecule (62). This circular molecule enters a recipient via conjugation and integrates into any of many sites on the recipient chromosome. Alternatively it may undergo intracellular transposition onto another replicon by a mechanism that appears to be the reciprocal of excision (20).

The larger transposons, such as Tn*5252* prefer to insert at a unique site (in *S. pneumoniae* and *S. gordonii*) by a site-specific integration mechanism (78). Unlike the Tn*916*-like transposons, there has been no evidence that excision of the Tn*5252*-class of elements results in a circular excision product during conjugal transposition. In a previous report, Guild *et al.* (31) stated that during filter mating between pneumococcal strains, conjugal transposition of Tn*5253* was not subjected to DNA restriction by *Dpn*II while transfer of the coresident conjugative plasmid, pIP501 was found to be sensitive to restriction and reduced 10^4 fold. They also showed that the transposon DNA is restricted *in vitro* by *Dpn*II implying that, as with the F plasmid in *E. coli,* transposon DNA is transferred and maintained as single-stranded DNA and escapes from restriction, whereas the plasmid replicon becomes double stranded after its transfer and is susceptible to restriction. This suggests an alternate mechanism of transposition for the Tn*5252*-class of transposons. This assumption must be experimentally investigated.

The two insertions at the left terminus of Tn*5252* (coordinate 3.1 and 5.5, Figures 9 and 39) interfere with the conjugal transfer of the transposon. The nucleotide sequence of this region (coordinates 1.7 to 8.0) contains at least five open-reading frames (ORFs). Of the five, three ORFs were

compared to other deduced amino acid sequences in the data bases. The two open reading frames, spanning the insertion sites, have deduced amino acid sequence with similarities to those of excisase and relaxase families of proteins. This indicates that they may play a similar role in the conjugal transposition of Tn*5252*. The other ORF that spans the left most end of Tn*5252* (coordinate 2.9) has a deduced amino acid sequences with significant similarities to the integrases of the staphylococcal temperate phage L54a (83).

On the basis of experimental results presented in this study with the limited information on the conjugal gene-transfer mechanism in gram-positive bacteria, it is difficult to draw a working conjugal-transposition model for the transposon Tn*5252*. However, experimental results presented in this study would provide an exciting area of study of molecular biology of gene transfer mediated by conjugative transposons among a diverse group of clinically important species of gram-positive bacteria. Further experiments should be designed to understand the physical and functional properties of these transfer-related genes and their products.

Initially, experiments could be designed to investigate the complementation group of the transfer-related genes. To do this, specific wild-type fragments would be cloned on a gram-positive or an *E. coli*-streptococcal shuttle vector and introduced into the various mutant strains for *in trans* complementation of the mutant genes. Following complementation analysis, the gene of interest would be produced in *E. coli* by using an expression vector for further analysis. The physical properties of the purified proteins could be determined by employing standard techniques including sodium dodecyl sulfate (SDS), polyacrylamide gel electrophoresis (PAGE), gel filtration, and isoelectric focusing gel.

CONCLUSION

Tn*5253*, originally found in *S. pneumoniae* BM6001, is a 65.5-kb conjugative transposon, carries resistances to chloramphenicol and tetracycline. It has been shown that the *tet* region of this conjugative transposon is itself an independent conjugative transposon, designated Tn*5251*. This novel transposon is capable of conjugal transposition in pneumococci, *S. pyogenes* and *S. gordonii*. *In vivo* deletion of Tn*5251* from within Tn*5253* did not affect the conjugal transposition properties of the remaining sequences. The *cat* determinant was still capable of conjugative transposition and was designated Tn*5252*. Thus, Tn*5253* is a composite element made up of two conjugative transposons, Tn*5252* and Tn*5251*.

Structural and functional similarities between Tn*5251* and other *tet* elements, such as Tn*916*, and Tn*1545*, suggest that they have originated from a common ancestor.

The observed functional and structural differences between Tn*5252*-like elements and the Tn*5251* class of transposons suggested that they may represent two distinct class of conjugative transposons.

Rather than being the prototype, the Tn*5251*-class of elements were possibly added later on to a preexisting element as supported by the absence of detectable homology between these elements and termini of the larger transposon.

Therefore, as the genetic and functional properties of Tn*5252* are found to be different from those of the smaller conjugative transposons, it is

probable that it will be found to be a prototype for the larger transposons such as Tn*5253* and Tn*3701*.

To obtain a functional map of Tn*5252*, a series of defined deletion and insertion mutations were produced within the transposon. The pneumococcal strains carrying these mutant derivatives of the transposons were tested for their ability to act as donors in filter-mating experiments with *S. pyogenes* recipients. Interruption of at least five regions did not affect the conjugal transposition functions whereas four of the nine insertion mutants and all six deletion mutants were found to be transfer impaired. Insertions in the middle of the transposon did not affect the transfer function of Tn*5252* suggested that this segment of DNA could serve as a target for integration of other mobile elements such as Tn*5251* and *cat* gene without affecting the transfer functions.

Studies of specific genes and their products at the molecular level will provide more information on the mechanism of transposition of these conjugative transposon.

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VITA 2

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